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COST IN U.S. DOLLARS                             SINCE FILE      TOTAL
                                                      ENTRY    SESSION
FULL ESTIMATED COST                                    0.21       0.21

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FILE 'EMBASE' ENTERED AT 11:16:25 ON 15 JUL 2004
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FILE 'BIOSIS' ENTERED AT 11:16:25 ON 15 JUL 2004
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FILE 'SCISEARCH' ENTERED AT 11:16:25 ON 15 JUL 2004

FILE 'CAPLUS' ENTERED AT 11:16:25 ON 15 JUL 2004

=> s "HVEM:Fc"
L1 16 "HVEM:FC"

=> dup remove l1
PROCESSING COMPLETED FOR L1
L2 5 DUP REMOVE L1 (11 DUPLICATES REMOVED)

=> d l2 1-5 cbib abs

L2 ANSWER 1 OF 5 CAPLUS COPYRIGHT 2004 ACS on STN
2001:781125 Document No. 135:343309 Ligand p30/LIGHT for HVEM (herpes virus entry mediator) and methods of therapeutic use. Ware, Carl F. (La Jolla Institute for Allergy and Immunology, USA). PCT Int. Appl. WO 2001079496 A2 20011025, 104 pp. DESIGNATED STATES: W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM; RW: AT, BE, BF, BJ, CF, CG, CH, CI, CM, CY, DE, DK, ES, FI, FR, GA, GB, GR, IE, IT, LU, MC, ML, MR, NE, NL, PT, SE, SN, TD, TG, TR. (English). CODEN: PIXXD2. APPLICATION: WO 2001-US11857 20010411. PRIORITY: US 2000-524325 20000313; US 2000-549096 20000412.

AB A novel polypeptide ligand, p30, for HVEM (herpes virus entry mediator) and functional variations and fragments thereof are provided. The HVEM ligand is isolated from II-23.D7 cell line, a human CD4+ T cell hybridoma. P30, which can be found as a membrane protein and can function as a cytokine, is also called LIGHT, because this polypeptide is homologous to Lymphotoxins, exhibits Inducible expression, and competes with HSV Glycoprotein D for HVEM, a receptor expressed by T lymphocytes. Because LIGHT can compete with HSV glycoprotein D for HVEM, homo-trimeric soluble forms of this polypeptide can be used to block the entry of herpesvirus into cells. P30 is useful for modulating immune responses and in inhibiting infection and/or subsequent proliferation by herpesvirus. LIGHT also bind to the lymphotoxin-β receptor (LTβR). The present invention is also based upon the discovery that HVEM polypeptides have an antagonistic effect on inflammation. In particular, HVEM fusion proteins are capable of inhibiting inflammation when administered to a subject. **HVEM-Fc** fusion proteins are also provided. Methods for treating subjects with lymphoid cell disorders, tumors, autoimmune diseases, inflammatory disorders of those having or suspected of having a herpes virus infection, utilizing p30 and the fusion proteins of the invention, are also provided.

L2 ANSWER 2 OF 5 MEDLINE on STN DUPLICATE 1
2000487952. PubMed ID: 11035077. Reciprocal expression of the TNF family receptor herpes virus entry mediator and its ligand LIGHT on activated T cells: LIGHT down-regulates its own receptor. Morel Y; Schiano de Colella J M; Harrop J; Deen K C; Holmes S D; Wattam T A; Khandekar S S; Truneh A; Sweet R W; Gastaut J A; Olive D; Costello R T. (Laboratoire d'Immunologie des Tumeurs, Departement d'Hematologie, Institut Paoli Calmettes, Universite de la Mediterranee, Marseille, France.) Journal of immunology (Baltimore, Md. : 1950), (2000 Oct 15) 165 (8) 4397-404. Journal code: 2985117R. ISSN: 0022-1767. Pub. country: United States. Language: English.

AB The TNF receptor (TNFR) family plays a central role in the development of the immune response. Here we describe the reciprocal regulation of the recently identified TNFR superfamily member herpes virus entry mediator

(HVEM) (TR2) and its ligand LIGHT (TL4) on T cells following activation and the mechanism of this process. T cell activation resulted in down-regulation of HVEM and up-regulation of LIGHT, which were both more pronounced in CD8(+) than CD4(+) T lymphocytes. The analysis of HVEM and LIGHT mRNA showed an increase in the steady state level of both mRNAs following stimulation. LIGHT, which was present in cytoplasm of resting T cells, was induced both in cytoplasm and at the cell surface. For HVEM, activation resulted in cellular redistribution, with its disappearance from cell surface. HVEM down-regulation did not rely on de novo protein synthesis, in contrast to the partial dependence of LIGHT induction. Matrix metalloproteinase inhibitors did not modify HVEM expression, but did enhance LIGHT accumulation at the cell surface. However, HVEM down-regulation was partially blocked by a neutralizing mAb to LIGHT or an **HVEM-Fc** fusion protein during activation. As a model, we propose that following stimulation, membrane or secreted LIGHT binds to HVEM and induces receptor down-regulation. Degradation or release of LIGHT by matrix metalloproteinases then contributes to the return to baseline levels for both LIGHT and HVEM. These results reveal a self-regulating ligand/receptor system that contributes to T cell activation through the interaction of T cells with each other and probably with other cells of the immune system.

L2 ANSWER 3 OF 5 MEDLINE on STN DUPLICATE 2

1998438532. PubMed ID: 9765287. Herpesvirus entry mediator ligand (HVEM-L), a novel ligand for HVEM/TR2, stimulates proliferation of T cells and inhibits HT29 cell growth. Harrop J A; McDonnell P C; Brigham-Burke M; Lyn S D; Minton J; Tan K B; Dede K; Spampanato J; Silverman C; Hensley P; DiPrinzio R; Emery J G; Deen K; Eichman C; Chabot-Fletcher M; Truneh A; Young P R. (Department of Molecular and Cellular Immunology, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406, USA.) Journal of biological chemistry, (1998 Oct 16) 273 (42) 27548-56. Journal code: 2985121R. ISSN: 0021-9258. Pub. country: United States. Language: English.

AB Herpesvirus entry mediator (HVEM), a member of the tumor necrosis factor (TNF) receptor family, mediates herpesvirus entry into cells during infection. Upon overexpression, HVEM activates NF-kappaB and AP-1 through a TNF receptor-associated factor (TRAF)-mediated mechanism. Using an **HVEM-Fc** fusion protein, we screened soluble forms of novel TNF-related proteins derived from an expressed sequence tag data base. One of these, which we designated HVEM-L, specifically bound to **HVEM-Fc** with an affinity of 44 nM. This association was confirmed with soluble and membrane forms of both receptor and ligand. HVEM-L mRNA is expressed in spleen, lymph nodes, macrophages, and T cells and encodes a 240-amino acid protein. A soluble, secreted form of the protein stimulates proliferation of T lymphocytes during allogeneic responses, inhibits HT-29 cell growth, and weakly stimulates NF-kappaB-dependent transcription.

L2 ANSWER 4 OF 5 MEDLINE on STN DUPLICATE 3

1998411370. PubMed ID: 9739048. LIGHT, a novel ligand for lymphotoxin beta receptor and TR2/HVEM induces apoptosis and suppresses in vivo tumor formation via gene transfer. Zhai Y; Guo R; Hsu T L; Yu G L; Ni J; Kwon B S; Jiang G W; Lu J; Tan J; Ugustus M; Carter K; Rojas L; Zhu F; Lincoln C; Endress G; Xing L; Wang S; Oh K O; Gentz R; Ruben S; Lippman M E; Hsieh S L; Yang D. (Human Genome Sciences, Inc., Rockville, Maryland 20850, USA.) Journal of clinical investigation, (1998 Sep 15) 102 (6) 1142-51. Journal code: 7802877. ISSN: 0021-9738. Pub. country: United States. Language: English.

AB LIGHT is a new member of tumor necrosis factor (TNF) cytokine family derived from an activated T cell cDNA library. LIGHT mRNA is highly expressed in splenocytes, activated PBL, CD8(+) tumor infiltrating lymphocytes, granulocytes, and monocytes but not in the thymus and the tumor cells examined. Introduction of LIGHT cDNA into MDA-MB-231 human breast carcinoma caused complete tumor suppression in vivo. Histological examination showed marked neutrophil infiltration and necrosis in LIGHT

expressing but not in the parental or the Neo-transfected MDA-MB-231 tumors. Interferon gamma (IFNgamma) dramatically enhances LIGHT-mediated apoptosis. LIGHT protein triggers apoptosis of various tumor cells expressing both lymphotoxin beta receptor (LTbetaR) and TR2/HVEM receptors, and its cytotoxicity can be blocked specifically by addition of a LTbetaR-Fc or a TR2/**HVEM-Fc** fusion protein.
However, LIGHT was not cytolytic to the tumor cells that express only the LTbetaR or the TR2/HVEM or hematopoietic cells examined that express only the TR2/HVEM, such as PBL, Jurkat cells, or CD8(+) TIL cells. In contrast, treatment of the activated PBL with LIGHT resulted in release of IFNgamma. Our data suggest that LIGHT triggers distinct biological responses based on the expression patterns of its receptors on the target cells. Thus, LIGHT may play a role in the immune modulation and have a potential value in cancer therapy.

L2 ANSWER 5 OF 5 CAPLUS COPYRIGHT 2004 ACS on STN
1997:220640 Document No. 126:208748 Cloning and expression of cDNA for herpes simplex virus cellular mediator HVEM and pharmaceuticals derived from the protein and cDNA. Spear, Patricia G.; Montgomery, Rebecca I. (Northwestern University, USA; Spear, Patricia G.; Montgomery, Rebecca I.). PCT Int. Appl. WO 9704658 A1 19970213, 57 pp. DESIGNATED STATES: W: CA, US; RW: AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE. (English). CODEN: PIXXD2. APPLICATION: WO 1996-US12374 19960726. PRIORITY: US 1995-509024 19950728.
AB The present invention provides isolated and purified polynucleotides that encode HVEM of mammalian origin, expression vectors containing those polynucleotides, host cells transformed with those expression vectors, a process of making HVEM using those polynucleotides and vectors, and isolated and purified HVEM. Antisense nucleic acids based on the cDNA and HVEM or HVEM derivs. may be used in pharmaceuticals. HeLa cell cDNA encoding HVEM was cloned and sequenced. Based on the DNA sequence anal. indicating that the product is a type I membrane glycoprotein with 3.5 Cys-rich repeats, HVEM is proposed to be a new member of the tumor necrosis factor/nerve growth factor receptor family. CHO-K1 and CHO-ST cell lines resistant to HSV-1 entry become significantly more susceptible when expressing the HVEM cDNA. Anti-HVEM antiserum protected these cells from infection, but the mechanism was not that of preventing binding to the cells. An **HVEM-Fc** fusion protein also inhibited infection of the HVEM-producing recombinant cells.

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=> s "LTbetaR:Fc"
L3          10 "LTBETAR:FC"

=> dup remove l3
PROCESSING COMPLETED FOR L3
L4            6 DUP REMOVE L3 (4 DUPLICATES REMOVED)

=> d l4 1-6 cbib abs
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L4 ANSWER 1 OF 6 MEDLINE on STN DUPLICATE 1
2002334128. PubMed ID: 12077273. Differing activities of homeostatic chemokines CCL19, CCL21, and CXCL12 in lymphocyte and dendritic cell recruitment and lymphoid neogenesis. Luther Sanjiv A; Bidgol Afshin; Hargreaves Diana C; Schmidt Andrea; Xu Ying; Paniyadi Jyothi; Matloubian Mehrdad; Cyster Jason G. (Howard Hughes Medical Institute and Department of Microbiology and Immunology, University of California, San Francisco, CA 94143-0414, USA.) Journal of immunology (Baltimore, Md. : 1950), (2002 Jul 1) 169 (1) 424-33. Journal code: 2985117R. ISSN: 0022-1767. Pub. country: United States. Language: English.
AB Despite their widespread expression, the in vivo recruitment activities of CCL19 (EBV-induced molecule 1 ligand chemokine) and CXCL12 (stromal cell-derived factor 1) have not been established. Furthermore, although CXCL13 (B lymphocyte chemoattractant) has been shown to induce lymphoid neogenesis through induction of lymphotoxin (LT)alpha1beta2, it is unclear

whether other homeostatic chemokines have this property. In this work we show that ectopic expression in pancreatic islets of CCL19 leads to small infiltrates composed of lymphocytes and dendritic cells and containing high endothelial venules and stromal cells. Ectopic CXCL12 induced small infiltrates containing few T cells but enriched in dendritic cells, B cells, and plasma cells. Comparison of CCL19 transgenic mice with mice expressing CCL21 (secondary lymphoid tissue chemokine) revealed that CCL21 induced larger and more organized infiltrates. A more significant role for CCL21 is also suggested in lymphoid tissues, as CCL21 protein was found to be present in lymph nodes and spleen at much higher concentrations than CCL19. CCL19 and CCL21 but not CXCL12 induced LTalpha1beta2 expression on naive CD4 T cells, and treatment of CCL21 transgenic mice with **LTbetaR-Fc** antagonized development of organized lymphoid structures. LTalpha1beta2 was also induced on naive T cells by the cytokines IL-4 and IL-7. These studies establish that CCL19 and CXCL12 are sufficient to mediate cell recruitment in vivo and they indicate that LTalpha1beta2 may function downstream of CCL21, CCL19, and IL-2 family cytokines in normal and pathological lymphoid tissue development.

L4 ANSWER 2 OF 6 MEDLINE on STN
2003190818. PubMed ID: 12110133. Multiple roles for tumor necrosis factor-alpha and lymphotoxin alpha/beta in immunity and autoimmunity. McDevitt Hugh; Munson Sibyl; Ettinger Rachel; Wu Ava. (Department of Microbiology and Immunology, Stanford University Medical Center, California 94305, USA.. hughmcd@stanford.edu) . Arthritis research, (2002) 4 Suppl 3 S141-52. Ref: 75. Journal code: 100913255. ISSN: 1465-9905. Pub. country: England: United Kingdom. Language: English.

AB Tumor necrosis factor (TNF)-alpha and lymphotoxin (LT) alpha/beta play multiple roles in the development and function of the immune system. This article focuses on three important aspects of the effects of these cytokines on the immune response and on autoimmunity. In several experimental systems (Jurkat T cells, murine T-cell hybridomas), TNF-alpha appears to cause a downregulation of signaling through the TCR, revealed by changes in calcium flux, activation of p21, p23 and ZAP70, and a decrease in nuclear activation of NF-kappaB. Previous and present results suggest that TNF-alpha interferes in some manner with signaling through the TCR, at a locus yet to be delineated. Transgenic expression of **LTbetaR-Fc** in nonobese diabetic (NOD) transgenic mice results in prevention of type 1 diabetes in NOD mice as long as the level of expression of the fusion protein (under the control of the cytomegalovirus promoter) remains above a level of 2-3 microg/ml. Once the expression levels of the fusion protein have dropped below this critical level, the diabetic process resumes and the animals become diabetic at 40-50 weeks of age, whereas nontransgenic littermates develop diabetes by 25-30 weeks of age. The paradoxical effects of neonatal TNF-alpha administration in NOD mice in increasing incidence of and hastening onset of type 1 diabetes, while neonatal anti-TNF administration completely prevents all signs of islet cell autoimmunity, are due partly to the low levels of CD4+CD25+ T cells in NOD mice. These low levels are reduced by a further 50% on neonatal administration of nontoxic levels of TNF-alpha. In contrast, neonatal administration of anti-TNF-alpha results in a dramatic increase in the levels of CD4+CD25+ regulatory T cells, to levels beyond those seen in wild-type untreated NOD mice. TNF-alpha and LTalpha/beta thus have pleomorphic regulatory effects on the development and expression of autoimmunity.

L4 ANSWER 3 OF 6 MEDLINE on STN DUPLICATE 2
2001320052. PubMed ID: 11390441. A critical role for lymphotoxin-beta receptor in the development of diabetes in nonobese diabetic mice. Ettinger R; Munson S H; Chao C C; Vadeboncoeur M; Toma J; McDevitt H O. (Basel Institute for Immunology, Basel CH-4005, Switzerland.) Journal of experimental medicine, (2001 Jun 4) 193 (11) 1333-40. Journal code: 2985109R. ISSN: 0022-1007. Pub. country: United States. Language: English.

AB To assess the role of lymphotoxin-beta receptor (LTbetaR) in diabetes

pathogenesis, we expressed an **LTbetaR-Fc** fusion protein in nonobese diabetic (NOD) mice. The fusion protein was expressed in the embryo, reached high levels for the first 2 wk after birth, and then declined progressively with age. High expression of **LTbetaR-Fc** blocked diabetes development but not insulitis. After the decline in chimeric protein concentration, mice became diabetic with kinetics similar to the controls. Early expression of fusion protein resulted in disrupted splenic architecture. However, primary follicles and follicular dendritic cells, but not marginal zones, developed in aged mice. Hence, LTbetaR signaling is required for diabetes development and regulates follicular and marginal zone structures via qualitatively or quantitatively distinct mechanisms.

L4 ANSWER 4 OF 6 BIOSIS COPYRIGHT 2004 BIOLOGICAL ABSTRACTS INC. on STN
2001:527466 Document No.: PREV200100527466. Lymphotoxins and cytomegalovirus cooperatively induce interferon-beta, establishing host-virus detente. Benedict, Chris A.; Banks, Theresa A.; Senderowicz, Lionel; Ko, Mira; Britt, William J.; Angulo, Ana; Ghazal, Peter; Ware, Carl F. [Reprint author]. Division of Molecular Immunology, La Jolla Institute for Allergy and Immunology, San Diego, CA, 92121, USA. carl_ware@liai.org. Immunity, (October, 2001) Vol. 15, No. 4, pp. 617-626. print.
ISSN: 1074-7613. Language: English.

AB Tumor necrosis factor (TNF)-related cytokines regulate cell death and survival and provide strong selective pressures for viruses, such as cytomegalovirus (CMV), to evolve counterstrategies in order to persist in immune-competent hosts. Signaling by the lymphotoxin (LT)-beta receptor or TNF receptor-1, but not Fas or TRAIL receptors, inhibits are cytopathicity and replication of human CMV by a nonapoptotic, reversible process that requires nuclear factor kappaB (NF-kappaB)-dependent induction of interferon-beta (IFN-beta). Efficient induction of IFN-beta requires virus infection and LT signaling, demonstrating the need for both host and viral factors in the curtailment of viral replication without cellular elimination. LTalpha-deficient mice and **LTbetaR-Fc** transgenic mice were profoundly susceptible to murine CMV infection. Together, these results reveal an essential and conserved role for LTs in establishing host defense to CMV.

L4 ANSWER 5 OF 6 MEDLINE on STN
1999138810. PubMed ID: 9973387. Lymphotoxin alphabeta is expressed on recently activated naive and Th1-like CD4 cells but is down-regulated by IL-4 during Th2 differentiation. Gramaglia I; Mauri D N; Miner K T; Ware C F; Croft M. (Divisions ofImmunochemistry and Molecular Immunology, La Jolla Institute for Allergy and Immunology, San Diego, CA 92121, USA.) Journal of immunology (Baltimore, Md. : 1950), (1999 Feb 1) 162 (3) 1333-8. Journal code: 2985117R. ISSN: 0022-1767. Pub. country: United States. Language: English.

AB Lymphotoxin (LT) is a cytokine that orchestrates lymphoid neogenesis and formation of germinal center reactions. LT exists as a membrane heterotrimer of alpha and beta subunits and is secreted as a homotrimer, LTalpha3. Using **LTbetaR.Fc**, expression of LTalphabeta on CD4 T cell subsets was investigated in a TCR transgenic model. LTalphabeta was evident 24-72 h after activation of naive T cells with specific Ag, and declined thereafter. Early expression was independent of IFN-gamma and IL-12, however, IL-12 prolonged expression. LTalphabeta was reinduced within 2-4 h after Ag restimulation, but declined by 24 h regardless of IL-12 or IFN-gamma priming. Exposure of naive T cells to IL-4 did not affect early LTalphabeta expression at 24 h, but resulted in subsequent down-regulation. IL-4-differentiated Th2 effectors did not re-express LTalphabeta, and LTalphabeta was transiently found on Th1 clones but not Th2 clones. LTalpha3 and TNF were immunoprecipitated from supernatants and lysates of IL-12 primed cells but not IL-4 primed cells. These studies demonstrate that LTalphabeta is expressed by activated naive CD4 cells, unpolarized IL-2-secreting effectors, and Th1 effectors. In contrast, loss of surface LTalphabeta and a lack of LTalpha3 and TNF secretion is associated with prior exposure to IL-4 and a Th2 phenotype.

L4 ANSWER 6 OF 6 MEDLINE on STN DUPLICATE 3
1998411370. PubMed ID: 9739048. LIGHT, a novel ligand for lymphotoxin beta receptor and TR2/HVEM induces apoptosis and suppresses in vivo tumor formation via gene transfer. Zhai Y; Guo R; Hsu T L; Yu G L; Ni J; Kwon B S; Jiang G W; Lu J; Tan J; Ugustus M; Carter K; Rojas L; Zhu F; Lincoln C; Endress G; Xing L; Wang S; Oh K O; Gentz R; Ruben S; Lippman M E; Hsieh S L; Yang D. (Human Genome Sciences, Inc., Rockville, Maryland 20850, USA.) Journal of clinical investigation, (1998 Sep 15) 102 (6) 1142-51. Journal code: 7802877. ISSN: 0021-9738. Pub. country: United States. Language: English.

AB LIGHT is a new member of tumor necrosis factor (TNF) cytokine family derived from an activated T cell cDNA library. LIGHT mRNA is highly expressed in splenocytes, activated PBL, CD8(+) tumor infiltrating lymphocytes, granulocytes, and monocytes but not in the thymus and the tumor cells examined. Introduction of LIGHT cDNA into MDA-MB-231 human breast carcinoma caused complete tumor suppression in vivo. Histological examination showed marked neutrophil infiltration and necrosis in LIGHT expressing but not in the parental or the Neo-transfected MDA-MB-231 tumors. Interferon gamma (IFNgamma) dramatically enhances LIGHT-mediated apoptosis. LIGHT protein triggers apoptosis of various tumor cells expressing both lymphotoxin beta receptor (LTbetaR) and TR2/HVEM receptors, and its cytotoxicity can be blocked specifically by addition of a **LTbetaR-Fc** or a TR2/HVEM-Fc fusion protein.
However, LIGHT was not cytolytic to the tumor cells that express only the LTbetaR or the TR2/HVEM or hematopoietic cells examined that express only the TR2/HVEM, such as PBL, Jurkat cells, or CD8(+) TIL cells. In contrast, treatment of the activated PBL with LIGHT resulted in release of IFNgamma. Our data suggest that LIGHT triggers distinct biological responses based on the expression patterns of its receptors on the target cells. Thus, LIGHT may play a role in the immune modulation and have a potential value in cancer therapy.

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=> s inflammation and HVEM
L5          24 INFLAMMATION AND HVEM

=> dup remove l5
PROCESSING COMPLETED FOR L5
L6          14 DUP REMOVE L5 (10 DUPLICATES REMOVED)

=> d l6 1-14 cbib abs
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L6 ANSWER 1 OF 14 CAPLUS COPYRIGHT 2004 ACS on STN DUPLICATE 1
2004:204306 Document No. 140:234408 Differentially expressed nucleic acids in human leukocytes useful for diagnosing or monitoring autoimmune and chronic inflammatory diseases. Wohlgemuth, Jay; Fry, Kirk; Woodward, Robert; Ly, Ngoc (USA). U.S. Pat. Appl. Publ. US 2004009479 A1 20040115, 484 pp., Cont.-in-part of U.S. Ser. No. 6,290. (English). CODEN: USXXCO. APPLICATION: US 2002-XC131827 20020424. PRIORITY: US 2001-PV296764 20010608; US 2001-6290 20011022; US 2002-131827 20020424.

AB Methods of diagnosing or monitoring an autoimmune or chronic inflammatory disease, particularly systemic lupus erythematosis (SLE) in a patient by detecting the expression level of one or more genes or surrogates derived therefrom in the patient are described. Diagnostic oligonucleotides for diagnosing or monitoring chronic inflammatory disease, particularly SLE infection and kits or systems containing the same are also described. Thus, over 8000 50-mer oligonucleotide microarray probes are designed from human leukocyte, plant, and viral genes identified by subtractive hybridization, sequencing, and database mining as candidates for human clin. conditions. Six hundred twenty-three cDNA sequences derived from human leukocytes were not homologous to UniGene sequences or sequences found in dbEST at the time they were search. The invention also provides lupus gene expression markers, PCR primers for each of the lupus markers, and surrogates for the lupus gene expression markers. [This abstract record is one of several

records for this document necessitated by the large number of index entries required to fully index the document and publication system constraints.].

L6 ANSWER 2 OF 14 MEDLINE on STN DUPLICATE 2

2004131603. PubMed ID: 15024715. Structural pathways for macromolecular and cellular transport across the blood-brain barrier during inflammatory conditions. Review. Lossinsky A S; Shivers R R. (Immunohistochemistry and Electron Microscopy Laboratories, Neural Engineering Program, Huntington Medical Research Institutes, Pasadena, California, USA.. ALossinsky@aol.com) . Histology and histopathology, (2004 Apr) 19 (2) 535-64. Journal code: 8609357. ISSN: 0213-3911. Pub. country: Spain. Language: English.

AB This review presents an overview of the highlights of major concepts involving the anatomical routes for the transport of macromolecules and the transmigration of cellular elements across the blood-brain barrier (BBB) during **inflammation.** The particular focus will include inflammatory leukocytes, neoplastic cells and pathogenic microorganisms including specific types of viruses, bacteria and yeasts. The experimental animal models presented here have been employed successfully by the authors in several independent experiments during the past twenty-five years for investigations of pathologic alterations of the BBB after a variety of experimentally induced injuries and inflammatory conditions in mammalian and non-mammalian animal species. The initial descriptions of endothelial cell (EC) vesicles or caveolae serving as mini-transporters of fluid substances essentially served as a springboard for many subsequent discoveries during the past half century related to mechanisms of uptake of materials into ECs and whether or not pinocytosis is related to the transport of these materials across EC barriers under normal physiologic conditions and after tissue injury. In the mid-1970's, the authors of this review independently applied morphologic techniques (transmission electron microscopy-TEM), in conjunction with the plant protein tracer horseradish peroxidase (HRP) to investigate macromolecular transport structures that increased after the brain and spinal cord had been subjected to a variety of injuries. Based on morphologic evidence from these studies of BBB injury, the authors elaborated a unique EC system of modified caveolae that purportedly fused together forming transendothelial cell channels, and later similar EC profiles defined as vesiculo-canalicular or vesiculo-tubular structures (VTS, Lossinsky, et al., 1999). These EC structures were observed in association with increased BBB permeability of tracers including exogenously injected HRP, normally excluded from the intercellular milieu of the CNS. Subsequent studies of non-BBB-type tumor ECs determined that the EC VTS and other vesicular structures were defined by others as vesiculo-vacuolar organelles (VVOs, Kohn et al., 1992; Dvorak et al., 1996). Collectively, these structures appear to represent a type of anatomical gateway to the CNS likely serving as conduits. However, these CNS conduits become patent only in damaged ECs for the passage of macromolecules, and purportedly for inflammatory and neoplastic cells as well (Lossinsky et al., 1999). In this review, we focus attention on the similarities and differences between caveolae, fused racemic vesicular bundles, endothelial tubules and channels (VTS and the VVOs) that are manifest in normal, non-BBB-type blood vessels, and in the BBB after injury. This review will present evidence that the previous studies by the authors and other researchers established a framework for subsequent transmission (TEM), scanning (SEM) and high-voltage electron microscopic (**HVEM**) investigations concerning ultrastructural, ultracytochemical and immunoultra-structural alterations of the cerebral ECs and the mechanisms of the BBB transport that occurs after CNS injury. This review is not intended to include all of the many observations that might be included in a general historical overview of the development of the EC channel hypothesis, but it will discuss several of the major contributions. We have attempted to present some of the structural evidence that supports our early contributions and those made by other investigators by highlighting major features of these EC structures that are manifest in the injured BBB. We have focused on currently established concepts and principles related to mechanisms for

the transendothelial transport of macromolecules after CNS injury and also offer a critical appraisal of some of this literature. Finally, we describe more recent concepts of transBBB avenues for viruses, including HIV-1, bacterial and mycotic organisms, as well as inflammatory and neoplastic cell adhesion and migration across the injured mammalian BBB. Data from studies of EC-related adhesion molecules, both from the literature and from the author's experimental results and observations made in other laboratories, as well as from personal communications underscore the importance of the adhesion molecules in facilitating the movement of leukocytic, neoplastic cell and human pathogens across the BBB during inflammatory and neoplastic events. Exciting, ongoing clinical trials are addressing possible therapeutic intervention in neuroinflammatory diseases, including multiple sclerosis, by blocking certain glycoprotein adhesion molecules before cells have the ability to adhere to the ECs and migrate across the BBB. Approaches whereby **inflammation** may be reduced or arrested using anti-adhesion molecules, by restructuring EC cytoskeletal, filamentous proteins, as well as remodeling cholesterol components of the modified VTS are discussed in the context of developing future therapies for BBB injury and **inflammation.** Understanding new concepts about the mechanism(s) by which inflammatory cells and a variety of pathogenic microorganisms are transported across the BBB can be expected to advance our understanding of fundamental disease processes. Taken together, the literature and the author's experiences during the past quarter of a century, will hopefully provide new clues related to the mechanisms of transendothelial cell adhesion and emigration across the injured BBB, issues that have been receiving considerable attention in the clinical arena. Learning how to chemically modulate the opening and/or closure of EC VTS and VVO structural pathways, or junctional complexes prior to cellular or microorganism adhesion and breaching the BBB presents challenging new questions in modern medicine. Future studies will be critically important for the development of therapeutic intervention in several human afflictions including traumatic brain and spinal cord injuries, stroke, cancer, multiple sclerosis and conditions where the immune system may be compromised including HIV infection, infantile and adult meningitis.

L6 ANSWER 3 OF 14 CAPLUS COPYRIGHT 2004 ACS on STN

2003:836779 Document No. 139:336920 Antibodies specific to TR2 protein for diagnosis, prognosis and treatment of cancer, **inflammation**, autoimmune disease, immunodeficiency and infection. Rosen, Craig A.; Ruben, Steven M. (Human Genome Sciences, Inc., USA). PCT Int. Appl. WO 2003086301 A2 20031023, 196 pp. DESIGNATED STATES: W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM; RW: AT, BE, BF, BJ, CF, CG, CH, CI, CM, CY, DE, DK, ES, FI, FR, GA, GB, GR, IE, IT, LU, MC, ML, MR, NE, NL, PT, SE, SN, TD, TG, TR. (English). CODEN: PIXXD2. APPLICATION: WO 2003-US10955 20030410. PRIORITY: US 2002-PV371722 20020412.

AB The present invention relates to antibodies and related mols. such as scFv that specifically bind to TR2 proteins, also known as TNFR-related 2, herpesvirus entry mediator (**HVEM**), or TRAF-associated receptor (ATAR). Such antibodies have uses, for example, in the prevention and treatment of cancers and other proliferative disorders, autoimmune disorders, immunodeficiencies and/or HSV infection. The invention also relates to nucleic acid mols. encoding anti-TR2 antibodies, vectors and host cells containing these nucleic acids, and methods for producing the same. The present invention relates to methods and compns. for preventing, detecting, diagnosing, treating or ameliorating a disease or disorder, especially cancer and other hyperproliferative disorders, comprising administering to an animal, preferably a human, an effective amount of one or more antibodies or fragments thereof, or related mols., that specifically bind to TR2.

L6 ANSWER 4 OF 14 SCISEARCH COPYRIGHT 2004 THOMSON ISI on STN
2004:139806 The Genuine Article (R) Number: 768BH. High levels of soluble herpes virus entry mediator in sera of patients with allergic and autoimmune diseases. Jung H W; La S J; Kim J Y; Heo S K; Kim J Y; Wang S; Kim K K; Lee K M; Cho H R; Lee H W; Kwon B; Kim B S; Kwon Y S (Reprint). Univ Ulsan, Immunomodulat Res Ctr, Ulsan 680749, South Korea (Reprint); Univ Ulsan, Dept Biol Sci, Ulsan 680749, South Korea; Immunom Inc, Ulsan 680749, South Korea; Indiana Univ, Sch Med, Dept Microbiol & Immunol, Indianapolis, IN 46202 USA; Seoul Natl Univ, Coll Dent, Dept Oral Microbiol, Seoul 110744, South Korea; Ulsan Univ Hosp, Dept Internal Med, Ulsan 682714, South Korea; Ulsan Univ Hosp, Dept Surg, Ulsan 682714, South Korea; Louisiana State Univ, Ctr Eye, Sch Med, New Orleans, LA 70112 USA. EXPERIMENTAL AND MOLECULAR MEDICINE (31 DEC 2003) Vol. 35, No. 6, pp. 501-508. Publisher: KOREAN SOC MED BIOCHEMISTRY MOLECULAR BIOLOGY. #812 KOFST, 635-4 YOKSAM-DONG KANGNAM-GU, SEOUL 135-703, SOUTH KOREA. ISSN: 1226-3613. Pub. country: South Korea; USA. Language: English.
ABSTRACT IS AVAILABLE IN THE ALL AND IALL FORMATS

AB Herpes virus entry mediator (**HVEM**) is a newly discovered member of the tumor necrosis factor receptor (TNFR) superfamily that has a role in herpes simplex virus entry, in T cell activation and in tumor immunity. We generated mAb against **HVEM** and detected soluble **HVEM** (SHVEM) in the sera of patients with various autoimmune diseases. **HVEM** was constitutively expressed on CD4(+) and CD8(+) T cells, CD19(+) B cells, CD14(+) monocytes, neutrophils and dendritic cells. In three-way MLR, mAb 122 and 139 were agonists and mAb 108 had blocking activity. An ELISA was developed to detect sHVEM in patient sera. sHVEM levels were elevated in sera of patients with allergic asthma, atopic dermatitis and rheumatoid arthritis. The mAbs discussed here may be useful for studies of the role of **HVEM** in immune responses. Detection of soluble **HVEM** might have diagnostic and prognostic value in certain immunological disorders.

L6 ANSWER 5 OF 14 MEDLINE on STN DUPLICATE 3
2003127996. PubMed ID: 12642898. Involvement of tumor necrosis factor receptor superfamily(TNFRSF) members in the pathogenesis of inflammatory diseases. Kwon Byungsuk; Kim Byung-Sam; Cho Hong Rae; Park Jeong-Euy; Kwon Byoung Se. (The Immunomodulation Research Center University of Ulsan, Ulsan 680-749, Korea.. bkwon@mail.ulsan.ac.kr) . Experimental & molecular medicine, (2003 Feb 28) 35 (1) 8-16. Ref: 71. Journal code: 9607880. ISSN: 1226-3613. Pub. country: Korea (South). Language: English.

AB Current therapies for autoimmune diseases are not cures but merely palliatives, aimed at reducing symptoms. For the most part, these treatments provide nonspecific suppression of the immune system and thus do not distinguish between a pathogenic autoimmune response and a protective immune response. Recently emerging evidence not only has indicated the involvement of members of the TNF receptor/ligand superfamilies but also has revealed exciting innovative strategies for the treatment of autoimmune diseases and other chronic inflammatory diseases without depressing the immune response in general. In this review, we will discuss the regulatory mechanisms of TNF receptor/ligand family members, such as **HVEM**/ LIGHT, 4-1BB/4-1BBL, and GITR/GITRL that regulate T and B cell functions and participate in the process of inflammatory diseases. We will also discuss how intervening in the costimulatory pathways mediated by these molecules might have some potential as a therapeutic approach to immune disorders.

L6 ANSWER 6 OF 14 CAPLUS COPYRIGHT 2004 ACS on STN
2002:716321 Document No. 137:246527 Multivalent MHC constructs: Immunoanalysis, diagnosis and therapy. Winther, Lars; Petersen, Lars Oestergaard; Buus, Soeren; Schoeller, Joergen; Ruub, Erik; Aamellem, Oeystein (Dako A/S, Den.; Dynal Biotech Asa). PCT Int. Appl. WO 2002072631 A2 20020919, 304 pp. DESIGNATED STATES: W: AE, AG, AL, AM, AT, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, CZ, DE, DE, DK, DK, DM, DZ, EC, EE, EE, ES, FI, FI, GB, GD, GE, GH, GM, HR,

HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW, AM, AZ, BY, KG; RW: AT, BE, BF, BJ, CF, CG, CH, CI, CM, CY, DE, DK, ES, FI, FR, GA, GB, GR, IE, IT, LU, MC, ML, MR, NE, NL, PT, SE, SN, TD, TG, TR. (English). CODEN: PIXXD2. APPLICATION: WO 2002-DK169 20020313. PRIORITY: DK 2001-435 20010314; DK 2001-436 20010314; DK 2001-441 20010314; US 2001-PV275470 20010314; US 2001-PV275448 20010314; US 2001-PV275447 20010314.

AB The authors disclose MHC mol. constructs (classical and non-classical) conjugated to soluble or insol. carriers wherein the affinity and avidity of the constructs exceed that of comparable MHC tetramers. In one example, the construct is comprised of biotinylated HLA-A2 bound to FITC-labeled streptavidin conjugated to soluble derivatized dextran. The above construct loaded with MART-1 or influenza virus peptides was shown to effect T-cell activation at a lower concentration than. Also comprised by the present invention is the sample-mounted use of MHC mols., MHC mol. multimers, and MHC mol. constructs.

L6 ANSWER 7 OF 14 CAPLUS COPYRIGHT 2004 ACS on STN
2002:107363 Document No. 136:149860 B7-H3 and B7-H4, novel immunoregulatory molecules. Chen, Lieping (Mayo Foundation for Medical Education and Research, USA). PCT Int. Appl. WO 2002010187 A1 20020207, 61 pp. DESIGNATED STATES: W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM; RW: AT, BE, BF, BJ, CF, CG, CH, CI, CM, CY, DE, DK, ES, FI, FR, GA, GB, GR, IE, IT, LU, MC, ML, MR, NE, NL, PT, SE, SN, TD, TG, TR. (English). CODEN: PIXXD2. APPLICATION: WO 2001-US41430 20010726. PRIORITY: US 2000-PV220991 20000727.

AB The invention provides novel B7-H3 and B7-H4 polypeptides useful for co-stimulating T cells, isolated nucleic acid mols. encoding them, vectors containing the nucleic acid mols., and cells containing the vectors. Also included are methods of making and using these co-stimulatory polypeptides.

L6 ANSWER 8 OF 14 CAPLUS COPYRIGHT 2004 ACS on STN
2001:781125 Document No. 135:343309 Ligand p30/LIGHT for **HVEM** (herpes virus entry mediator) and methods of therapeutic use. Ware, Carl F. (La Jolla Institute for Allergy and Immunology, USA). PCT Int. Appl. WO 2001079496 A2 20011025, 104 pp. DESIGNATED STATES: W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM; RW: AT, BE, BF, BJ, CF, CG, CH, CI, CM, CY, DE, DK, ES, FI, FR, GA, GB, GR, IE, IT, LU, MC, ML, MR, NE, NL, PT, SE, SN, TD, TG, TR. (English). CODEN: PIXXD2. APPLICATION: WO 2001-US11857 20010411. PRIORITY: US 2000-524325 20000313; US 2000-549096 20000412.

AB A novel polypeptide ligand, p30, for **HVEM** (herpes virus entry mediator) and functional variations and fragments thereof are provided. The **HVEM** ligand is isolated from II-23.D7 cell line, a human CD4+ T cell hybridoma. P30, which can be found as a membrane protein and can function as a cytokine, is also called LIGHT, because this polypeptide is homologous to Lymphotoxins, exhibits Inducible expression, and competes with HSV Glycoprotein D for **HVEM**, a receptor expressed by T lymphocytes. Because LIGHT can compete with HSV glycoprotein D for **HVEM**, homo-trimeric soluble forms of this polypeptide can be used to block the entry of herpesvirus into cells. P30 is useful for modulating immune responses and in inhibiting infection and/or subsequent proliferation by herpesvirus. LIGHT also bind to the lymphotoxin-β receptor (LTβR). The present invention is also based upon the

discovery that **HVEM** polypeptides have an antagonistic effect on **inflammation.** In particular, **HVEM** fusion proteins are capable of inhibiting **inflammation** when administered to a subject. **HVEM**-Fc fusion proteins are also provided. Methods for treating subjects with lymphoid cell disorders, tumors, autoimmune diseases, inflammatory disorders of those having or suspected of having a herpes virus infection, utilizing p30 and the fusion proteins of the invention, are also provided.

L6 ANSWER 9 OF 14 CAPLUS COPYRIGHT 2004 ACS on STN
2001:598037 Document No. 135:179726 Identification of a novel domain in the tumor necrosis factor receptor family that mediates pre-ligand receptor assembly and function. Lenardo, Michael J.; Chan, Francis Ka-ming; Siegel, Richard M. (Government of the United States of America as Represented by the Secretary, Department of Health and Human Services, USA). PCT Int. Appl. WO 2001058953 A2 20010816, 77 pp. DESIGNATED STATES: W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM; RW: AT, BE, BF, BJ, CF, CG, CH, CI, CM, CY, DE, DK, ES, FI, FR, GA, GB, GR, IE, IT, LU, MC, ML, MR, NE, NL, PT, SE, SN, TD, TG, TR. (English). CODEN: PIXXD2. APPLICATION: WO 2001-US4125 20010209. PRIORITY: US 2000-PV181909 20000211.

AB The authors disclose the identification and characterization of an amino acid sequence termed PLAD (pre-ligand assembly domain) found in the extracellular domains of the TNF receptor superfamily. In one example, self-association of the p60 and p80 receptor monomers was demonstrated to occur in the absence of the TNF-α ligand. In a second example using wild-type and engineered constructs of CD95, the apoptotic signaling function was shown to correlate with the ability to self-associate independent of ligand binding.

L6 ANSWER 10 OF 14 CAPLUS COPYRIGHT 2004 ACS on STN
2001:338564 Document No. 134:348630 New members of the TRAF (tumor necrosis factor receptor-associated factor) protein family with possible therapeutic uses. Zapata, Juan M.; Reed, John C. (The Burnham Institute, USA). PCT Int. Appl. WO 2001032696 A2 20010510, 156 pp. DESIGNATED STATES: W: AE, AG, AL, AM, AT, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, CZ, DE, DE, DK, DK, DM, DZ, EE, EE, ES, FI, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM; RW: AT, BE, BF, BJ, CF, CG, CH, CI, CM, CY, DE, DK, ES, FI, FR, GA, GB, GR, IE, IT, LU, MC, ML, MR, NE, NL, PT, SE, SN, TD, TG, TR. (English). CODEN: PIXXD2. APPLICATION: WO 2000-US30533 20001103. PRIORITY: US 1999-434784 19991105.

AB In accordance with the present invention, there are provided novel TRAF-Protein-Binding-Domain polypeptides (TPBDs). The invention also provides nucleic acid mols. encoding TPBDs, vectors containing these nucleic acid mols. and host cells containing the vectors. The invention also provides antibodies that can specifically bind to invention TPBDs. Such TPBDs and/or anti-TPBD antibodies are useful for discovery of drugs that suppress autoimmunity, **inflammation**, allergy, allograft rejection, sepsis, and other diseases. Characterization of the proteins is reported and their interaction of other members of the family. A reporter gene assay for measuring their effects on NF-κB activity is described.

L6 ANSWER 11 OF 14 BIOSIS COPYRIGHT 2004 BIOLOGICAL ABSTRACTS INC. on STN
2001:276732 Document No.: PREV200100276732. Functional analysis of **HVEM**, a member of TNFR family, by using a transgenic mice expressing soluble form of **HVEM.** Inobe, Manabu [Reprint author]; Uenishi, Tomoko [Reprint author]; Murakami, Masaaki [Reprint author];

Uede, Toshimitsu [Reprint author]. Div. Mol. Immunol., Inst. Genet. Med., Hokkaido Univ., Kita-15, Nishi-7, Kita-ku, Sapporo, 060-0815, Japan. FASEB Journal, (March 7, 2001) Vol. 15, No. 4, pp. A352. print.
Meeting Info.: Annual Meeting of the Federation of American Societies for Experimental Biology on Experimental Biology 2001. Orlando, Florida, USA. March 31-April 04, 2001.
CODEN: FAJOEC. ISSN: 0892-6638. Language: English.

AB **HVEM** was first identified as a mediator of herpes virus entry into cell. It binds with envelope glycoprotein D expressed on surface of herpes virus. We cloned a **HVEM** cDNA through Differential Display of mRNA expression using spleens of allograft recipients: one showing acute rejection and the other showing long-term acceptance of grafts. TNF/TNFR pathways are known to be involved in the regulation of **inflammation**, septic shock and generation of lymph nodes, thus it is expected that **HVEM** may be implicated in the regulation of immune system. To further analyze the molecular mechanisms for the involvement of **HVEM** in immune system, we prepared soluble form of **HVEM** protein as a fusion molecule consisting of extracellular domain of **HVEM** and IgG-Fc portion (HVEMIg). We found that HVEMIg could significantly inhibit the LPS-induced lymphocyte proliferation, while the same concentration of HVEMIg showed relatively weak inhibitory effect on mixed lymphocyte reaction. Furthermore, we generated transgenic mice expressing HVEMIg under the regulation of CAG promoter. We established 5 lineage of HVEMIg transgenic mouse. The significant levels of serum HVEMIg (>10 mug/ml) was detected in those transgenic mouse. Nevertheless serum HVEMIg was not detectable in control littermate. The T-cell dependent antibody production was significantly suppressed in those transgenic mice, while T-cell independent antibody response was not significantly different between transgenic mice and control littermate. To analyze if the generation of lymphoid organs is impaired in transgenic mice, homozygous mice is being generated, so that the high levels of serum HVEMIg can be expected. Detailed histological analysis is on going.

L6 ANSWER 12 OF 14 BIOSIS COPYRIGHT 2004 BIOLOGICAL ABSTRACTS INC. on STN
1999:438044 Document No.: PREV199900438044. A homogenous 384-well high throughput screen for novel tumor necrosis factor receptor: Ligand interactions using time resolved energy transfer. Moore, K. J.; Turconi, S.; Miles-Williams, A.; Djaballah, H.; Hurskainen, P.; Harrop, J.; Murray, K. J.; Pope, A. J. [Reprint author]. SmithKline Beecham Pharmaceuticals, New Frontiers Science Park (North) Third Avenue, Harlow, CM195AW, UK. Journal of Biomolecular Screening, (Aug., 1999) Vol. 4, No. 4, pp. 205-214. print.
ISSN: 1087-0571. Language: English.

AB The herpes virus entry mediator (**HVEM**) receptor and its ligand, **HVEM**-L, are involved in both herpes simplex virus type-1 (HSV-1) herpes simplex virus type-2 (HSV-2) infection, and in T-cell activation such that antagonists of this interaction are expected to have utility in viral and inflammatory diseases. In this report we describe the configuration of a homogeneous 384-well assay based on time-resolved energy transfer from a europium chelate on the **HVEM** receptor to an allophycocyanin (APC) acceptor on the ligand. Specific time resolved emission from the acceptor is observed on receptor:ligand complex formation. The results of various direct and indirect labeling strategies are described. Several assay optimization experiments were necessary to obtain an assay that was robust to automation and file compound interference while sensitive to the effect of potential inhibitors. The signal was stable for more than 24 h at room temperature using the Eu3+ chelates, suggesting no dissociation of the lanthanide ion. The 384-well assay was readily automated and was able to identify more than 99.5% of known positive controls in the validation studies successfully. Screening identified both a series of known potent inhibitors and several structural classes of hits that readily deconvoluted to yield single compound inhibitors with the desired functional activity in secondary biological assays. The equivalence of the data in 384- and 1536-well formats

indicates that routine implementation of 1536-well chelate-based energy transfer screening appears to be primarily limited by liquid handling rather than detection issues.

L6 ANSWER 13 OF 14 MEDLINE on STN DUPLICATE 4

91304320. PubMed ID: 2072867. Mechanisms of inflammatory cell attachment in chronic relapsing experimental allergic encephalomyelitis: a scanning and high-voltage electron microscopic study of the injured mouse blood-brain barrier. Lossinsky A S; Pluta R; Song M J; Badmajew V; Moretz R C; Wisniewski H M. (Department of Pathological Neurobiology, New York State Institute for Basic Research in Developmental Disabilities, Staten Island 10314.) Microvascular research, (1991 May) 41 (3) 299-310. Journal code: 0165035. ISSN: 0026-2862. Pub. country: United States. Language: English.

AB Brain and spinal cord blood vessels from mice subjected to chronic relapsing experimental allergic encephalomyelitis were examined by scanning (SEM) and high-voltage electron microscopy (**HVEM**). SEM analysis of veins and venules from affected tissue regions demonstrated inflammatory cells (ICs), primarily lymphocytes or monocytes, attached to the luminal endothelial cell (EC) surface adjacent to the junctional complexes. In transverse section these cells were shown by **HVEM** to extend and to insert filopodia (lymphocytes) or flap-like lamellapodia (monocytes) into the luminal EC surfaces. Affected ECs often expressed increased microvillar projection as well as parajunctional crater-like structures on their luminal surfaces. Based on scanning and high-voltage electron microscopy, we present morphological evidence that some populations of sensitized ICs do not penetrate the EC junctions initially during EC attachment but instead insert pseudopodial projections into specialized openings in the ECs that are formed in response to chronic **inflammation**.

L6 ANSWER 14 OF 14 MEDLINE on STN DUPLICATE 5

84045737. PubMed ID: 6356473. **Inflammation** with restricted lysosomal proteolysis during early ascites carcinoma invasion of mouse parietal peritoneum. A medium and high-voltage electron microscopic and cytochemical study. Parsons D F; Marko M; Wansor K J. Tissue & cell, (1983) 15 (4) 499-507. Journal code: 0214745. ISSN: 0040-8166. Pub. country: ENGLAND: United Kingdom. Language: English.

AB A carcinoma invasion system (Krebs-2 and Ehrlich tetraploid ascites tumors invading mouse peritoneum) was studied by high-voltage electron microscope (**HVEM**) stereoscopy, conventional (medium voltage) electron microscopy (MVEM), and cytochemistry. Tumor cells entered areas of peritoneum (mainly parietal) only where mesothelial cells were damaged and where there was **inflammation** of the underlying stroma. The initial invasion was different from that of most other invading carcinomas in that there was minimal breakdown of basal lamina and collagen. Neither tumor cells, inflammatory leukocytes nor peritoneal fibroblasts showed significant secondary lysosome production or release of intracellular or extracellular acid phosphatase. Morphological and cytochemical criteria suggest that in some invading carcinomas, as with non-tumor migrating cells such as leukocytes, widespread proteolysis due to diffusion of proteases is not a prerequisite for invasion of stromal connective tissue.

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L10 ANSWER 1 OF 4 CAPLUS COPYRIGHT 2004 ACS on STN
2003:242032 Document No. 138:253721 Application of LIGHT and herpes simplex virus entry mediator in therapy. **Ware, Carl** (USA). U.S. Pat. Appl. Publ. US 2003060605 A1 20030327, 76 pp., Cont.-in-part of U.S. Ser. No. 549,096. (English). CODEN: USXXCO. APPLICATION: US 2001-967604 20010928. PRIORITY: US 1997-PV51964 19970707; US 1997-898234 19970730; US 2000-549096 20000412.

AB The author discloses p30, or LIGHT, a cytokine ligand for the herpes virus entry mediator, HVEM. A soluble construct of LIGHT was shown to inhibit cytomegalovirus infection and to trigger apoptosis of HT29 tumor cells. Soluble HVEM fusion proteins are also provided and shown to exhibit antiinflammatory activity in delayed-type hypersensitivity and **rheumatoid arthritis** models.

L10 ANSWER 2 OF 4 EMBASE COPYRIGHT 2004 ELSEVIER INC. ALL RIGHTS RESERVED. on STN
2003232429 EMBASE Introduction: The TNF superfamily. **Ware C.F.**.
C.F. Ware, Division of Molecular Immunology, La Jolla Inst. for Allerg./Immunol., 10355 Science Center Drive, San Diego, CA 92121, United States. cware@liai.org. Cytokine and Growth Factor Reviews 14/3-4 (181-184) 2003.
ISSN: 1359-6101. CODEN: CGFRFB. Pub. Country: United Kingdom. Language: English.

L10 ANSWER 3 OF 4 CAPLUS COPYRIGHT 2004 ACS on STN
2001:781125 Document No. 135:343309 Ligand p30/LIGHT for HVEM (herpes virus entry mediator) and methods of therapeutic use. **Ware, Carl F.** (La Jolla Institute for Allergy and Immunology, USA). PCT Int. Appl. WO 2001079496 A2 20011025, 104 pp. DESIGNATED STATES: W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM; RW: AT, BE, BF, BJ, CF, CG, CH, CI, CM, CY, DE, DK, ES, FI, FR, GA, GB, GR, IE, IT, LU, MC, ML, MR, NE, NL, PT, SE, SN, TD, TG, TR. (English). CODEN: PIXXD2. APPLICATION: WO 2001-US11857 20010411. PRIORITY: US 2000-524325 20000313; US 2000-549096 20000412.

AB A novel polypeptide ligand, p30, for HVEM (herpes virus entry mediator) and functional variations and fragments thereof are provided. The HVEM ligand is isolated from II-23.D7 cell line, a human CD4+ T cell hybridoma. P30, which can be found as a membrane protein and can function as a cytokine, is also called LIGHT, because this polypeptide is homologous to Lymphotoxins, exhibits Inducible expression, and competes with HSV Glycoprotein D for HVEM, a receptor expressed by T lymphocytes. Because LIGHT can compete with HSV glycoprotein D for HVEM, homo-trimeric soluble forms of this polypeptide can be used to block the entry of herpesvirus into cells. P30 is useful for modulating immune responses and in inhibiting infection and/or subsequent proliferation by herpesvirus. LIGHT also bind to the lymphotoxin-β receptor (LTβR). The present invention is also based upon the discovery that HVEM polypeptides have an antagonistic effect on inflammation. In particular, HVEM fusion proteins are capable of inhibiting inflammation when administered to a subject. HVEM-Fc fusion proteins are also provided. Methods for treating subjects with lymphoid cell disorders, tumors, autoimmune diseases, inflammatory disorders of those having or suspected of having a herpes virus infection, utilizing p30 and the fusion proteins of the invention, are also provided.

L10 ANSWER 4 OF 4 MEDLINE on STN DUPLICATE 1

90150775. PubMed ID: 2302843. Enhanced cytotoxicity in the rheumatoid joint. LaCour E G; Grayson M H; **Ware C F**; Pope R M. (Northwestern University School of Medicine, Department of Medicine, Chicago, Illinois.) Clinical immunology and immunopathology, (1990 Mar) 54 (3) 431-41. Journal code: 0356637. ISSN: 0090-1229. Pub. country: United States. Language: English.

AB The cytotoxic cytokines, tumor necrosis factor-alpha or cachectin and lymphotoxin (LT), are mediators of bone resorption and of inflammation and may have relevance in **rheumatoid arthritis.** Using mononuclear cells (MC) isolated from matched peripheral blood (PB) and synovial fluid (SF) of 13 patients with **rheumatoid arthritis**, we examined the generation of cytotoxic activity in a bioassay capable of detecting both TNF and LT. Synovial fluid mononuclear cells (MC) released significantly more cytotoxic activity than did matched PBMC, both spontaneously and following activation with phytohemagglutinin P (PHA). When PB and SFMC were stimulated with the combination of PHA plus phorbol-12-myristate acetate (PMA), the resulting culture supernatants possessed comparable cytotoxic activity. Neutralization studies employing anti-cytokine antibodies indicated that TNF represented 43 and 59% of the cytotoxic activity in the PHA plus PMA-induced culture supernatants from PB and SF, respectively. Since no inhibition was noted with antibodies to LT, the nature of the remaining approximately 50% of the cytotoxic activity was not determined. In PB and SF culture supernatants, obtained both spontaneously and following PHA activation, the concentration of TNF measured by ELISA significantly correlated with the level of cytotoxicity. As with the cytotoxic activity, the concentration of TNF was greater in the PHA-stimulated supernatants from SF than from PB. These observations suggest that TNF in the SF may contribute to the inflammation and bone destruction observed in **rheumatoid arthritis.**

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Immunity

Volume 8, Issue 1 , 1 January 1998, Pages 21-30

doi:10.1016/S1074-7613(00)80455-0 Cite or Link Using DOI

Article

LIGHT, a New Member of the TNF Superfamily, and Lymphotoxin α Are Ligands for Herpesvirus Entry Mediator

Davide N. Mauri[1], Reinhard Ebner[5], Rebecca I. Montgomery[2], Kristine D. Kochel[1], Timothy C. Cheung[1], Guo-Liang Yu[5], Steve Ruben[5], Marianne Murphy[6], Roselyn J. Eisenberg[3], Gary H. Cohen[4], Patricia G. Spear[2] and Carl F. Ware[1, 1]

[1] Division of Molecular Immunology, La Jolla Institute for Allergy and Immunology, San Diego, California 92121, USA

[2] Department of Microbiology-Immunology, Northwestern University Medical School, Chicago, Illinois 60611, USA

[3] Department of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6002, USA

[4] Department of Microbiology, School of Dental Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104-6002, USA

[5] Department of Protein Therapeutics, Human Genome Sciences, Rockville, Maryland 20850, USA

[6] Department of Cell Biology, Human Genome Sciences, Rockville, Maryland 20850, USA

Received 12 October 1997; Revised 16 December 1997. Available online 3 October 2000.

Abstract

Herpes simplex virus (HSV) 1 and 2 infect activated T lymphocytes by attachment of the HSV envelope glycoprotein D (gD) to the cellular herpesvirus entry mediator (HVEM), an orphan member of the tumor necrosis factor receptor superfamily. Here, we demonstrate that

HVEM binds two cellular ligands, secreted lymphotoxin α (LTα) and LIGHT, a new member of the TNF superfamily. LIGHT is a 29 kDa type II transmembrane protein produced by activated T cells that also engages the receptor for the LT$\alpha\beta$ heterotrimer but does not form complexes with either LTα or LTβ. HSV1 gD inhibits the interaction of HVEM with LIGHT, and LIGHT and gD interfere with HVEM-dependent cell entry by HSV1. This characterizes herpesvirus gD as a membrane-bound viokine and establishes LIGHT-HVEM as integral components of the lymphotoxin cytokine-receptor system.

[8] These authors made equal contributions.

Further Reading

The GenBank accession number for LIGHT is AF036581.

Corresponding author: Carl F. Ware, 619-678-4655 (phone), 619-558-3595 (fax)

Immunity
Volume 8, Issue 1 , 1 January 1998, Pages 21-30

☐ 1: J Immunol. 1993 Dec 1;151(11):6602-7. Related Articles, Links

Influence of a recombinant human soluble tumor necrosis factor receptor FC fusion protein on type II collagen-induced arthritis in mice.

Wooley PH, Dutcher J, Widmer MB, Gillis S.

Department of Internal Medicine, Wayne State University School of Medicine, Detroit, MI 48201.

A recombinant human TNF receptor Fc fusion protein (rhuTNFR:Fc) was assessed for antiarthritic activity using murine type II collagen-induced arthritis in mice. DBA/1 mice were immunized with bovine type II collagen and treated with rhuTNFR:Fc either from day 21 to day 28 (preventative protocol), or after disease onset for fourteen days (therapeutic protocol). Control mice received either sterile saline or human serum albumin injections. rhuT-NFR:Fc treatment significantly reduced both the incidence and the severity of collagen-induced arthritis in the preventative protocol. Mice receiving rhuTNFR:Fc therapeutically progressed to less severe disease than did control animals, and the arthritis index in rhuTNFR:Fc treated mice was significantly lower than the index in control mice from 7.5 weeks after treatment. The antibody response to collagen was significantly reduced by treatment with rhuTNFR:Fc in both the preventative and therapeutic protocols. No difference was observed in the proliferative response to type II collagen or Con A, but the response to LPS was significantly lower in rhuTNFR:Fc treated mice at the conclusion of both the preventative and therapeutic trials. The results suggest that rhuTNFR:Fc may have both immunosuppressive and antiarthritic properties in this experimental model, and may represent a useful approach to the treatment of autoimmune arthritis.

PMID: 8245488 [PubMed - indexed for MEDLINE]

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1: Scand J Immunol. 1992 Jul;36(1):49-56. Related Articles, Links

Adjuvant composition determines the induction of type II collagen-induced arthritis.

Ellis JS, Chain BM, Cooke A, Ibrahim MA, Katz DR.

Department of Biology, University College London, UK.

In this study we have investigated the influence of adjuvant composition on the development of collagen-induced arthritis and of anti-collagen type II specific B- and T-cell responses following immunization with type II collagen. DBA/1 mice immunized with bovine collagen type II emulsified in complete Freund's adjuvant (CFA) containing Mycobacterium tuberculosis strain H37Ra developed footpad swelling indicative of arthritis. Animals immunized with collagen type II plus CFA containing Mycobacterium butyricum, or incomplete Freund's adjuvant showed no significant increase in footpad width. Induction of anti-CII specific T-cell proliferation was also dependent upon immunization with CII plus CFA containing M. tb H37RA. In contrast, ovalbumin-reactive T-cell proliferation was unaffected by the species of mycobacteria, indicating that the difference in adjuvant activity of the mycobacterial species is specific for anti-collagen type II T-cell responses. Antibody response to collagen type II, unlike T-cell responses, was not significantly different using the two adjuvants. This study therefore demonstrates that murine collagen-induced arthritis requires immunization with collagen type II together with complete Freund's adjuvant containing Mycobacterium tuberculosis H37RA. Since only this combination of antigen and adjuvant induces detectable arthritis and T-cell responses against collagen type II, while antibody synthesis does not have such stringent adjuvant requirements, this suggests that the development of the full pattern of the collagen-induced arthritis disease requires synergistic activation of both humoral and cell-mediated responses.

PMID: 1615283 [PubMed - indexed for MEDLINE]

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J Biol Chem, Vol. 275, Issue 15, 11121-11129, April 14, 2000

Discrete Signaling Regions in the Lymphotoxin-β Receptor for Tumor Necrosis Factor Receptor-associated Factor Binding, Subcellular Localization, and Activation of Cell Death and NF-κB Pathways*

Walker R. Force‡, Alison A. Glass, Chris A. Benedict, Timothy C. Cheung, Juan Lama, and Carl F. Ware§

From the Division of Molecular Immunology, La Jolla Institute for Allergy and Immunology, San Diego, California 92121

ABSTRACT

Lymphotoxin-β receptor (LTβR), a member of the tumor necrosis factor receptor superfamily, is essential for the development and organization of secondary lymphoid tissue. Wild type and mutant LTβR containing successive truncations of the cytoplasmic domain were investigated by retrovirus-mediated gene transfer into HT29.14s and in 293T cells by transfection. Wild type receptors accumulated in perinuclear compartments and enhanced responsiveness to ligand-induced cell death and ligand-independent activation of NFκB p50 dimers. Coimmunoprecipitation and confocal microscopy mapped the TRAF3 binding site to amino acids PEEGDPG at position 389. However, LTβR truncated at position Pro[379] acted as a dominant positive mutant that down-modulated surface expression and recruited TRAF3 to endogenous LTβR. This mutant exhibited ligand-independent cell death and activated NF-κB p50 dimers. By contrast, truncation at Gly[359] created a dominant-negative mutant that inhibited ligand-induced cell death and activation of NF-κB p50/p65 heterodimers. This mutant also blocked accumulation of wild type receptor into perinuclear compartments, suggesting subcellular localization may be crucial for signal transduction. A cryptic TRAF-independent NF-κB activating region was identified. These mutants define discrete subregions of a novel proline-rich domain that is required for subcellular localization and signal transduction by the LTβR.

INTRODUCTION

TOP
ABSTRACT
INTRODUCTION
MATERIALS AND METHODS
RESULTS
DISCUSSION
REFERENCES

The lymphotoxin β (LTβ)[1] receptor (LTβR), a member of the tumor necrosis factor receptor (TNFR) superfamily, has emerged as a signaling system required for organization of lymphoid tissue (for reviews, see Refs. 1 and 2). The LTβR binds two distinct but related ligands, the cell surface form of LT (3) and LIGHT (4). Surface LT is composed of two subunits, LTα and LTβ, arranged as a heterotrimers of either LTα1β2 (major form) or LTα2β1 stochiometry (5). The LTβ subunit, a type II transmembrane protein, provides the membrane anchor for the ligand and the specificity for binding the LTβR. The LTα subunit contributes primarily to the conformation of the heterotrimer (6) but can also form homotrimers that bind the two TNF receptors, TNFR1 (55-60 kDa; CD120a) and TNFR2 (80 kDa; CD120b). The second LTβR ligand, LIGHT, a recently identified member of the TNF superfamily (4), forms homotrimers and interacts with another TNFR family member, the herpesvirus entry mediator (HVEM or HveA) (7), which also binds LTα.

Although these ligands show significant cross-receptor specificity, each cytokine-receptor system plays distinct physiologic roles. Based on gene deletion studies, LTβR, but not TNFR, is required for the differentiation of secondary lymph organs, Peyer's patches, and lymph nodes (8). More recent evidence indicates that progenitor cells crucial for the development of natural killer cells and dendritic cell compartmentalization require the LTαβ-LTβR system (9-11). LTβR signaling also acts in concert with the TNF/TNFR1 system for the organization of peripheral lymphoid tissue during immune responses (12-15).

Signal transduction by the TNF receptors is initiated by the binding of specific trivalent ligands that induce aggregation of the receptors, which in turn recruit cytosolic proteins involved in the propagation of signals (16). LTβR interacts with TNF receptor-associated factors (TRAFs), a family of zinc RING finger proteins with a C-terminal region that binds directly to the cytoplasmic tail of LTβR and related receptors, such as CD40, CD30, and TNFR80 (17, 18). LTβR binds TRAF2, -3, -4, and -5, but not TRAF6 (19-22). Binding of soluble recombinant LTα1β2 or anti-LTβR antibodies rapidly induces the formation of a stable complex between TRAF3 and LTβR (19). Forced overexpression of these receptors leads to aggregation and activation of signaling pathways independent of ligand, indicating the presence of regulatory mechanisms that normally limit receptor expression or spontaneous aggregation. In cell culture models, signaling through LTβR induces cell death of certain adenocarcinoma tumor cells (23) and gene expression by activation of the p50/p65 form of nuclear factor κB (NF-κB) (19, 24), a transcription factor involved in controlling expression of proinflammatory molecules, including chemokines (25) and integrins (26, 27), and protection of cells from apoptotic death (28). Cell death and NF-κB pathways bifurcate at the level of LTβR-TRAF binding, since TRAF3 mutants block cell death signaling but not NF-κB activation (19, 29). TRAF2, -5, and -6 activate the NF-κB pathway by members of the TNFR superfamily, and a common binding site for TRAF2, -3, and -5, the PVQET sequence, has been identified in CD40 (30, 31), but this site is not readily apparent in the LTβR. Identification of the

regions in LTβR involved in binding TRAF proteins will aid in understanding the mechanisms of signal propagation. Furthermore, it is unclear whether TRAFs mediate all of the signaling activities of this receptor, because recent findings show that mice with gene deletions in TRAF2, -3, or -5 contain a normal complement of lymph nodes (32-34).

We have identified the structural regions of the LTβR required for TRAF binding and initiation of signaling that activates cell death and gene transcription pathways. A panel of LTβR mutants was characterized that reveal several novel features of LTβR signaling including three discrete regions within a short proline-rich sequence that control TRAF binding and receptor compartmentalization and regulate cell death and NF-κB activation. These mutants should provide useful tools for dissecting the molecular components and pathways involved in physiologic roles dependent on the LTβR.

MATERIALS AND METHODS

Cells and Reagents-- Recombinant human TNF (35) and soluble LT α1β2 (36) produced with a truncated version of LTβ lacking the cytosolic and transmembrane domains were provided by Jeffrey Browning (Biogen, Inc.). Mouse anti-Fas monoclonal antibody (mAb) CH11 (IgM) was obtained from MBL (Nagoya, Japan). M2 and M5 anti-Flag (IgG_1) mAb were obtained from Sigma. The anti-c-Myc monoclonal antibody 9E10 (IgG_1) was obtained from BABCO. Mouse anti-LTβR antibodies, BDA8 (IgG_1) and BK11 (IgG_1) were provided by Biogen, Inc. HT29.14s is a clone of the HT29 adenocarcinoma cell line sensitive to death-inducing effects of LT and related cytokines (23). Human embryonic kidney-293 cell line and its derivative 293T cells, which express the SV40 large T antigen, were obtained from the American Type Culture Collection. All cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum. All lines routinely tested negative for mycoplasm using a PCR-based assay, the Mycoplasma Primer Set (Stratagene, La Jolla, CA).

Plasmid Construction-- Retroviral plasmids directing the expression of the C-terminal truncated mutants of LTβR were constructed by PCR using c-Myc-LTβR-pBABE as a template (29). All deletion mutants were constructed using the common 5'-primer containing a *Bgl*II site, 5'-GACGAGAGATCTCTGGCTTCAGGAGCTGAATA-3'. A different 3'-primer was used for each deletion mutant. Each 3'-primer includes a stop codon and a *Sal*I site. The 3'-primers used were as follows: Δ418, 5'-GGAACGC**GTCGAC**TTAGTGCTCTGTCTCCGCTAGGTGCCAA-3'; Δ410, 5'-GGAACGC**GTCGAC**TTAAGCCTTGCCATCTTCCTGGTGGGGTGTA-3'; Δ403, 5'-GGAACGC**GTCGAC**TTAGGGTGTAGAGAGCCCGGGAGGGCCA-3'; Δ396, 5'-GGAACGC**GTCGAC**TTAGCCAGGGTCCCCCTCTTCGGGAAT-3'; Δ389, 5'-GGAACGC**GTCGAC**TTAAATGGGGTATGGAGGTTCGGGGGTA-3'; Δ379, 5'-GGAACGC**GTCGAC**TTAGAGGTCTCCAGGACCCGGTGGT-3'; Δ369, 5'-GGAACGC**GTCGAC**TTATACTGGTCCATTGTAGATGTA-3'; Δ359, 5'-GGAACGC**GTCGAC**TTAAGTGATAGTCATAGACCCGCCGGTGA-3'; Δ345, 5'-

GGAACGC**GTCGAC**TTAACCGTGGGCCACCTGGCTCT-3'.

PCR was performed under the following conditions (Perkin-Elmer 9600 thermocycler): an initial 4 min at 94 °C and then 30 cycles of 30 s at 94 °C, 30 s at 60 °C, and 80 s at 72 °C followed by a final cycle for 10 min at 72 °C. PCR products were generated using 30 ng of template plasmid and 5 units of *Pfu* DNA polymerase (Stratagene, La Jolla, CA). PCR products were purified using the Wizard PCR DNA Purification System (Promega, Madison, WI), digested with *Bgl*II and *Sal*I, gel-purified and ligated into the *Bam*HI and *Sal*I sites of pBABEpuro. The integrity of the c-Myc-LTβR coding region in each of the pBABEpuro deletion constructs was confirmed by sequencing both strands (ABI PRISM Dye Termination Cycle Sequencing kit) with an automated sequencer (ABI PRISM 310 genetic analyzer).

Moloney retroviral vectors were produced by transfection of the ΦNX amphotrophic packaging cell line with the desired pBABE-derived construct as described (29). For production of control virus, ΦNX cells were transfected with the empty pBABEpuro vector. Virus-containing supernatants were harvested after 48 h of transfection. Infection of the HT29.14s and 293 cell lines was performed as described previously (29). Infected cells were selected in puromycin, and all assays described were performed within 1-2 weeks following selection.

Flow Cytometric Analysis-- HT29.14s cells were detached from the culture vessel using 20 mM EDTA in phosphate-buffered saline (PBS), washed once in PBS, and then incubated with mouse isotype IgG control antibody, mouse anti-c-Myc epitope (9E10, IgG_1), or anti-human LTβR (BKA11, IgG_1) at 10 μg/ml in 50 μl/well in cold binding buffer (PBS with 2% fetal bovine serum, 0.05% sodium azide) for 30 min on ice. After washing three times with cold binding buffer, 50 μl of goat $F(ab')_2$ anti-mouse IgG conjugated to R-phycoerythrin (Southern Biotechnology Associates, Inc.) was added to wells and incubated for 30 min on ice. Cells were then washed as above and analyzed by flow cytometry (FACScan, Becton Dickenson).

Immunoprecipitation and Western Blot Analysis-- To detect TRAF3 binding to LTβR in HT29.14s cells or 293T cells, Western blot analysis was performed as described previously (19). Briefly, HT29.14s cell monolayers containing 10^7 cells were treated with 1 nM recombinant soluble LTα1β2 for 15 min at 37 ° C, or 2 × 10^5 293T cells transfected 48 h previously were extracted in buffer containing 1% Triton X-100, 0.15 M NaCl, 10 mM Tris, pH 7.4, and protease inhibitors) and subjected to immunoprecipitation with goat anti-LTβR IgG. After SDS-polyacrylamide gel electrophoresis, the gel was blotted onto polyvinylidene difluoride membrane, and TRAF3 was detected with a rabbit anti-TRAF3 antiserum (H122; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or mouse anti-FLAG (Sigma) or anti-c-Myc (9E10) mAb. Immune complexes were detected with goat anti-rabbit IgG or goat anti-mouse IgG peroxidase using an ECL detection kit (Amersham Pharmacia Biotech). The TRAF3 antiserum was used at a 1:1000 dilution and showed no cross-reactivity in Western blots with TRAF1, -2, or -5. Whole cell lysates were prepared by treating cell pellets in 1% SDS buffer with 3% mercaptoethanol, 50 mM Tris buffer, pH 6.8, for analysis by SDS-polyacrylamide gel electrophoresis and Western blot. Molecular mass markers (Bio-Rad) were phosphorylase *b* (106 kDa), bovine serum albumin (81 kDa), ovalbumin (47.5 kDa), carbonic anhydrase (35 kDa), and soybean trypsin inhibitor (28.2 kDa).

Cell Viability Assay-- Cell death in response to cytokines or antibodies was determined by a mitochondria dye reduction assay using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, which detects viable cells as described (37). After 3 days, the percentage of cell viability was calculated as a ratio of the dye absorbance (570 nm) by cells cultured with cytokines or antibodies to medium alone. After 3 days in culture, the A_{570} for individual lines in medium alone ranged from 1.1 to 1.6. Statistical analysis for calculations of IC_{50} and significance was calculated with GraphPad Prism and Instat software (San Diego, CA). Data are presented as the mean ± S.D. of quadruplicate wells.

NF-κB Activation-- NF-κB DNA binding interactions were performed by a electrophoretic gel shift assay as described (38) with modification (20) using the κB site in the human immunodeficiency virus-1 enhancer. The composition of the activated NF-κB complex was examined by supershift analysis with antiserum to Rel family members (Santa Cruz Biotechnology).

NF-κB-dependent transcription was measured using a luciferase reporter construct (39). 293T cells seeded at 5×10^5 cells/35-mm well were transfected by the $CaPO_4$ method. Briefly, 250 µl of precipitate containing 4 µg of LTβR expression plasmid and 0.25 µg of a NF-κB-luciferase reporter plasmid containing two copies of the consensus κB binding motif was added to the cells for an overnight incubation. Cells were washed once, and fresh growth medium was added for a 24-h incubation. Cells were extracted in 250 µl of lysis buffer (25 mM Tris-phosphate, pH 7.8, 2 mM dithiothreitol, 2 mM EDTA, 10% glycerol, 1% Triton X-100), and nuclei were removed by centrifugation at 7000 × *g*. Luciferase activity was detected in 50 µl of cell lysate using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA), which automatically injects luciferase assay buffer (20 mM Tricine, 1.07 mM $(MgCO_3)_4$, 2.67 mM $MgSO_4$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.27 mM coenzyme A, 0.47 mM sodium luciferin) and ATP (200 mM, pH 7.0). All samples were measured in duplicate.

Immunofluoresence Confocal Microscopy-- Twenty-four hours post-transfection, 293T cells were seeded in eight-well chamber slides (Lab-Tek) at 3×10^4 cells/well and cultured for 18-36 h at 37 °C. For staining, wells were washed twice with PBS, fixed for 10 min at room temperature in freshly prepared 2% paraformaldhyde in PBS, pH 7.0, washed twice with PBS, and then permeabilized in methanol for 2 min at room temperature. Cells were washed in PBS and then blocked for 10 min at room temperature in PBS containing 3% BSA. Polyclonal goat anti-LTβR TgG (19), diluted to a final concentration of 20 µg/ml, and mouse anti-FLAG M2 to detect TRAF3 were diluted in PBS containing 3% BSA and 0.2% Triton X-100 (PBS/BSA/Triton). Primary antibodies were added to the wells to a final volume of 120 µl/well and incubated in a humidified chamber at room temperature for 1 h. Wells were then washed three times in PBS/BSA/Triton buffer. Fluorescein isothiocyanate-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories) in combination with Texas Red-conjugated donkey anti-goat IgG (Jackson ImmunoResearch Laboratories), were diluted to a final concentration of 1:200 in PBS/BSA/Triton in a final volume of 120 µl/well. Slides were incubated in a humidified chamber at room temperature in the dark for 1 h and then washed three times in PBS/BSA/Triton. The slides were mounted in 80% glycerol in PBS, sealed, and kept at 4 °C in the dark for 1-7 days before visualization. Cells were observed with a Bio-Rad MRC-1024 confocal microscope with a krypton/argon ion laser and a 60× Nikon objective. Images were acquired using the LaserSharp

operation system and were analyzed and manipulated in Adobe PhotoShop. Empty vector-transfected cells or cells stained with normal goat serum or mouse IgG isotype control were used for negative controls. Neither control exhibited background staining. Representative staining patterns were based on counting 200 cells.

LTβR Structural Prediction-- Secondary structural prediction for the LTβR cytoplasmic domain was compiled by the Protein Sequence Analysis System (available on the World Wide Web) (40). The analysis was conducted on a subsequence of the LTβR, residues Thr^{242}-Asp^{435} C-terminal of the transmembrane domain, using a type-1 discrete state-space model. The model assumes that the cytoplasmic domain is monomeric, single-domain, globular, and water-soluble and folds into well defined structural domains that do not pack against a hydrophobic surface.

RESULTS

▲ TOP
▲ ABSTRACT
▲ INTRODUCTION
▲ MATERIALS AND METHODS
▪ RESULTS
▼ DISCUSSION
▼ REFERENCES

Structural Requirements for LTβR Expression and Recruitment of TRAF3-- The cytoplasmic domain of LTβR is 194 amino acids in length and is predicted to belong to the α-β structural superclass (probability = 0.9797) (Fig. 1, *A* and *B*). The polypeptide is predicted to emerge from the membrane as a stretch of ~120 residues that fold into three discrete helices, interspersed by β strands, which precedes a proline-rich stretch (38%) of 36 residues (Pro^{367}-Pro^{403}), likely to assume an elongated or kinked pedicle. The pedicle is followed by another β-strand conformation and a fourth helix at the C terminus. Glutathione *S*-transferase fusion proteins of the LTβR cytoplasmic domain previously indicated that the binding site(s) for TRAF2, -3, and -5 and Hepatitis C virus core protein are all located within residues 338-395, which spans the β-strand and pedicle regions (Ref. 41 and data not shown).

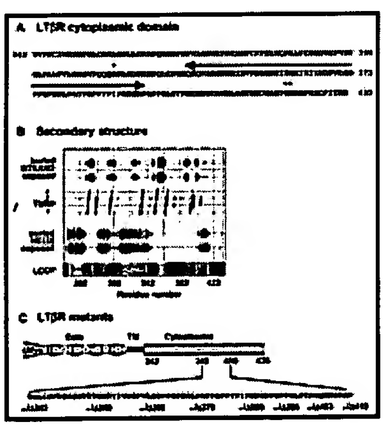

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Fig. 1. Structural features of the LTβR and mutants. *A*, sequence of the human LTβR cytoplasmic tail. The *arrow* indicates the region responsible for TRAF and hepatitis C virus core protein interactions. Putative protein kinase C (*) and protein kinase A (**) phosphorylation sites are shown. *B*, predicted secondary structure. Contour map of the predicted folds of the LTβR cytoplasmic domain as calculated by the Protein Sequence Analysis System. The *contour lines* show probability increments of 0.1. *C*, LTβR mutants. Mutants were constructed in a retrovirus expression vector by systematically deleting amino acid residues from the C-terminal cytoplasmic tail of the receptor. Each of these mutants contains an intact extracellular (*Ecto*) and transmembrane (*TM*) domain with an N-terminal c-Myc epitope tag. The LTβR deletions are indicated by Δ followed by the initial deleted amino acid.

A series of mutants were constructed to determine the subregions of the LTβR involved in TRAF

binding and cellular responses. LTβR mutants were made that successively truncate the C terminus through the pedicle and into the β-strand region and incorporate an N-terminal c-Myc epitope tag to distinguish mutant from endogenous receptor (Fig. 1C). HT29.14s cells trans-infected with retrovirus vectors expressing the c-Myc-LTβR deletion mutants revealed striking differences in cell surface expression (Fig. 2). Cells expressing wild type c-Myc-LTβR showed an ~2-3-fold increase over endogenous levels of surface LTβR as estimated from the difference in specific fluorescence staining between anti-c-Myc and anti-LTβR mAb used at saturating levels. Deletion through the C-terminal 36 residues (Δ389) showed no significant change in surface expression; however, the Δ379 mutant expressed little or no staining by anti-c-Myc. Furthermore, ~90% of the endogenous LTβR was also lost from the surface of Δ379 mutant-expressing cells. No evidence was obtained that shedding accounted for the decrease in LTβR expression on the cell surface, indicating that the protein is probably retained intracellular. By contrast, deletion of a further 10 residues (Δ369) led to increased expression on the cell surface of mutant and endogenous LTβR. The Δ359 mutant exhibited an ~5-fold increase in cell surface expression relative to the Δ369 mutant and ~3-fold above wild type receptor.

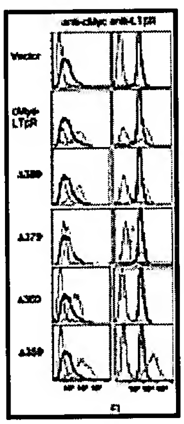

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Fig. 2. Cell surface expression of the c-Myc-LTβR deletion mutants in HT29.14s. HT29.14s cells were infected with retrovirus directing the expression of the wild type c-Myc-LTβR (*WT*), the indicated c-Myc-LTβR deletion mutant, or empty vector (*vector*). After selection, cells were stained with either mouse anti-c-Myc (9E10, IgG_1), anti-LTβR (BKA11, IgG_1), or control mouse IgG and detected with phycoerythrin-conjugated goat anti-mouse IgG. Cells were transinfected with the indicated LTβR-expressing vector and stained with control mouse IgG (*thin line*) or infected with the control vector (*thick dark line*) or with the indicated LTβR vector (*gray thick line*) and then stained with either anti-c-Myc (*left column*) or anti-LTβR (*right column*). Each histogram represents analysis of 5×10^3 cells.

In unmodified HT29.14s cells, TRAF3 co-immunoprecipitates with the LTβR after brief treatment of cells with ligand (19). In contrast, precipitation with anti-c-Myc revealed that TRAF3 was specifically associated with wild type c-Myc-LTβR independently of LTα1β2 (Fig. 3A). This result demonstrates that the modest overexpression of c-Myc-LTβR in this cell type is sufficient for ligand-independent TRAF3 recruitment. The Δ415 and Δ403 mutants also associated with TRAF3; however, TRAF3 binding was absent in the Δ345, Δ359, and Δ369 mutants. The Δ415 and Δ403 mutants also associated with TRAF3. However, the Δ379 mutant co-immunoprecipitated with a species of TRAF3 that migrated as a doublet of a lower molecular mass (~48-55 kDa), indicating that TRAF3 is specifically affected by the Δ379 mutation. The nature of this form of TRAF3 is unknown, but it could represent a proteolytic fragment among other possibilities. The analysis of TRAF3 binding was extended to HEK293 cells, including two additional mutants, Δ396 and Δ389, which showed that TRAF3 binding was specifically lost in Δ389

mutant (Fig. 3*B*). This result locates the TRAF3 binding site to residues ^{389}PEEGDP.

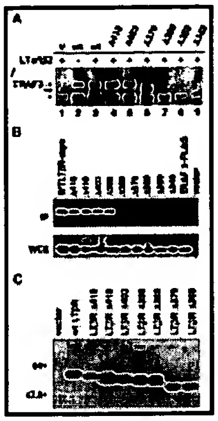

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Fig. 3. TRAF3 recruitment to the LTβR. *A*, TRAF3 association with LTβR in transinfected HT29.14s cells. HT29.14s cells infected with empty vector (*V*) or infected with virus directing the expression of c-Myc-LTβR wild type protein (*wt*) or one of the indicated c-Myc-LTβR deletion mutants were treated with LTα1β2 (1 nM) for 15 min prior to harvest. Cell lysates were prepared, immunoprecipitated with the anti-c-Myc antibody (9E10), and immunoblotted with an anti-TRAF3 polyclonal antibody (H122). The major TRAF3-specific band is indicated by an *arrow* to the *left*. The *lower arrow* to the *left* indicates two smaller TRAF3-specific bands observed only in c-Myc-LTβR-Δ379-expressing cells. The *asterisk* indicates a nonspecific band that reacted with the TRAF3 antiserum. *B*, TRAF3 binding site defined in 293T cells. 293T cells were transfected with TRAF3-FLAG expression vector and either a control retrovirus expression vector (*V*), vector directing the expression of c-Myc-LTβR-wild type protein (*wt*), or vector directing the expression of one of the indicated c-Myc-LTβR deletion mutants. c-Myc-LTβR was immunoprecipitated (*IP*) with polyclonal goat anti-LTβR from cell lysates and Western blotted for the presence of TRAF3 with mouse monoclonal anti-FLAG (M2). *Lower panel*, equivalent protein (40 μg) from whole cell extracts (*WCE*) of the transfected cells was subjected to Western blot analysis with mouse monoclonal anti-FLAG (M2) as a control for transfection of TRAF3. *C*, expression of LTβR mutants in HEK293 cells. Cells were transfected with c-Myc-LTβR mutants, and 48 h later the cells were lysed and immunoprecipated with goat anti-LTβR and Western blotted with mouse anti-c-Myc (9E10).

Expression of LTβR in HEK293 cells monitored by Western blot (Fig. 3*C*) revealed a single 69-kDa band for wild type receptor and proportionally smaller forms through mutant Δ403. Mutants Δ396 and Δ389 were resolved as a tight doublet in which the smaller form became predominant in mutants Δ379 and Δ369. This suggests that post-translation modification (*e.g.*, glycosylation) of the receptor was affected by the Δ379 mutation. The Δ359 and Δ345 mutants were not detected by blotting, although expression was readily detected on the cell surface by fluorescence staining, indicating that the overall abundance of these two mutants was decreased.

The loss of endogenous LTβR from the surface of HT29 cells and the coimmunoprecipitation of LTβR and TRAF3 in the absence of ligand prompted us to investigate the subcellular location of these mutants. LTβR and TRAF3 were investigated in the 293T cell line, which does not express detectable cell surface LTβR. Following transfection of the LTβR mutants into 293T cells, fluorescence staining analysis showed a pattern of expression similar to transinfected HT29.14s or HEK293 cells (data not shown). Confocal imaging revealed that the majority of the wild type LTβR and mutants through Δ369 accumulated as large clusters in perinuclear compartments (Fig. 4, *a-f*), and the diminished staining of cells that were fixed but not permeabilized indicated an intracellular location (data not shown). By contrast, Δ359 exhibited a diffuse, primarily surface-staining pattern, indicating that ^{360}NIYIYNGPVL^{369} is crucial for localization to these vesicles (Fig. 4*g*). TRAF3 expressed by itself

exhibited a diffuse cytoplasmic staining in 293T cells, but when coexpressed with wild type LTβR, it localized to the same perinuclear compartments, a pattern that was not altered by deletion of the LTβR through Δ396 (Fig. 4, *h-j*). As expected, TRAF3 failed to co-localize with the Δ389 mutant and was dispersed throughout the cytosol; however, LTβR remained in the perinuclear compartment (Fig. 4, *k-n*). Together, these results indicate that the TRAF3 binding site is defined by the Δ389 mutant, and this region is distinct from the region (Δ359) controlling subcellular compartmentalization of the LTβR. Interestingly, lysosome markers, cathepsin D or LAMP-1, and the endoplasmic reticulum marker AP-1 did not co-localize with the LTβR (data not shown). This result indicates that these LTβR/TRAF3-associated vesicles are unlikely to represent obstructed ER due to overexpression, nor do they appear to be a degradative end point. Further characterization of the subcellular compartments containing LTβR is in progress.

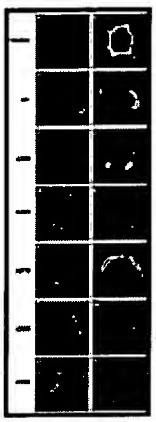

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Fig. 4. Co-localization of LTβR and TRAF3. 293T cells were transfected with a total of 2 μg of DNA/35-mm well as follows. *a* and *h*, empty vector; *b* and *i*, wild type LTβR; *c* and *j*, Δ396; *d* and *k*, Δ389; *e* and *l*, Δ379; *f* and *m*, Δ369; *g* and *n*, Δ359. Cells were cotransfected with empty vector (*panels a-g*) or with TRAF3-FLAG (*panels h-n)*. LTβR was stained with goat anti-LT βR and TRAF3-FLAG with mouse anti-FLAG M2. Texas Red-conjugated donkey anti-goat IgG (*red*) and fluorescein isothiocyanate-conjugated donkey anti-mouse IgG (*green*) were used to visualize their respective antigens. *Yellow* indicates colocalization.

That the Δ379 mutant failed to co-localize or bind directly to TRAF3 in 293T cells but co-immunoprecipitated with TRAF3 in HT29.14s cells indicates that an indirect mechanism allows TRAF3 to associate with the Δ379 mutant. A likely possibility is that Δ379 mutant associates with endogenous LTβR, which is complexed with TRAF3. This predicts that the Δ379 mutant should coimmunoprecipitate in a complex with wild type LTβR and TRAF3. To test if this association occurs, 293T cells were cotransfected with c-Myc-LTβRΔ379, TRAF3-FLAG, and wild type LTβR lacking an epitope tag. As predicted, the lysates subjected to immunoprecipitation with anti-c-Myc showed specific co-immunoprecipitation of TRAF3 by its association with c-Myc-LTβRΔ379 only in the presence of wild type LTβR (Fig. 5*A*). This association was also visualized by confocal microscopy (Fig. 5*B*), where TRAF3-FLAG colocalized with Δ379 in the perinuclear compartment only in the presence of wild type LTβR. We further utilized this transfection system to analyze the effect of the Δ359 dominant negative mutant. Confocal microscopy revealed that not only does the presence of Δ359 inhibit TRAF3 from colocalizing with wild type LTβR, but Δ359 also inhibited the accumulation of wild type receptor in the perinuclear compartments.

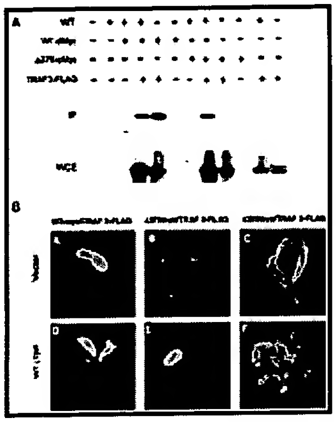

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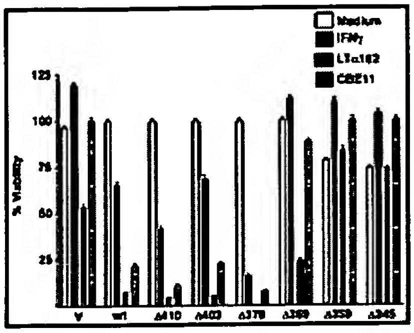
Fig. 5. Effect of dominant positive and negative mutants on compartmentalization of LTβR and TRAF3. *A*, the dominant positive Δ379 mutant indirectly associates with TRAF3 through wild type LTβR. 293T cells were transfected with the indicated combinations of empty vector, wild type LTβR untagged, wild type c-Myc-LTβR, c-Myc-Δ379, and TRAF3-FLAG, and 36 h after transfection cell lysates were prepared and immunoprecipitated with anti-c-Myc (9E10) and then Western blotted with rabbit anti-TRAF3 (H122). *Lower panel*, as a control for TRAF3-FLAG transfection, whole cell lysates were analyzed on a separate blot with anti-FLAG. *B*, compartmentalization of LTβR. 293T cells were transfected with the indicated combination of plasmids indicated on the *left* of each *row* and *above* each *column*. LTβR was detected with mouse anti-c-Myc (9E10) and donkey anti mouse IgG conjugated to Texas Red; TRAF3-FLAG was detected with goat anti-FLAG and donkey anti-goat fluorescein isothiocyanate.

The Effect of Dominant Positive and Negative Mutants of LTβR on Cell Death Signaling-- HT29 cells expressing the c-Myc-LTβR deletion mutants were treated with LTα1β2 or anti-LTβR mAb, (CBE11), and cell viability was determined after 3 days. In this model, treatment of the cells with IFN-γ is essential for apoptotic cell death induced by LTα1β2, TNF, or Fas (23, 46). Additionally, the anti-LTβR mAb CBE11 added in the soluble phase is normally not directly cytotoxic to HT29.14s cells unless combined with an additional anti-LTβR antibody (5). Treatment with IFN-γ of HT29.14s cells transduced with empty vector resulted in slight growth enhancement when compared with cells in medium, although together with LTα1β2 (1 nM) it induced a 50% decrease in cell viability, whereas LT βR antibody was not cytotoxic (Fig. 6). By contrast, HT29.14s cells expressing wild type c-Myc-LTβR responded to IFN-γ treatment in the absence of LTα1β2 with ~30% loss of cell viability when compared with medium-treated controls. However, a substantial decrease in cell viability occurred following treatment with LTα1β2 or anti-LTβR mAb. The Δ410 and Δ403 mutants responded to LTα1β2 treatment similar to wild type LTβR. In contrast, cells expressing Δ379 were exquisitely sensitive to treatment with IFN-γ alone, displaying a >85% loss in cell viability. The effect of IFN-γ and anti-LTβR mAb was lost with a further 10-residue deletion (Δ369), indicating that the sequence ^{369}LGGPPGPGDL^{378} is required for ligand-independent cell death response to IFN-γ. Indeed, the Δ369 mutant displayed a phenotype similar to the cells transduced with empty vector control, suggesting that this mutant is inactive and that the cell death response due to ligand alone occurs via endogenous LTβR. This is supported by the previous finding that Δ369 itself does not bind TRAF3. In contrast to the Δ369 mutant, Δ359 and Δ345 mutants were nonresponsive to LTα1β2 (Fig. 6).

Fig. 6. . Affect of c-Myc-LTβR expression on cell death. HT29.14s cells were infected with empty vector (*V*) or infected with virus directing the expression of the full-length c-Myc-LTβR-wild type protein (*wt*) or one of the indicated c-Myc-LTβR deletion mutants. Cells expressing the indicated



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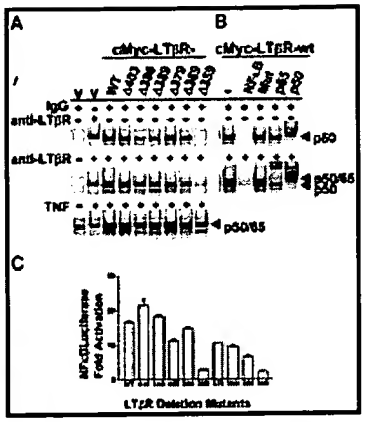
c-Myc-LTβR proteins were cultured in microtiter wells in medium (*white bars*) or medium with IFN-γ (80 units/ml), with LTα1β2 (1 nM) and IFN-γ, or with anti-LTβR monoclonal antibody CBE11 (1 µg/ml) and IFN-γ. After 72 h, cells were assayed for cell viability by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide staining. Cell viability was calculated as a percentage of medium-treated HT29.14s cells that were infected with empty vector virus.

The ligand-independent cell death induced by Δ379, together with the finding that this mutant down-regulates endogenous LTβR and indirectly co-immunoprecipitated with TRAF3, defines the behavior predicted for a dominant positive mutant. On the other side, the Δ359 mutant acts as a dominant negative, perhaps due to increased cell surface expression relative to wild type receptor or by blockade of wild type receptor trafficking into perinuclear compartments or association of wild type LTβR with TRAF3.

Ligand-dependent and -independent Activation of p50 and p50/p65 NF-κB Complexes by the LTβR--That NF-κB activation by the LTβR is not inhibited by dominant negative TRAF3 mutants indicates that signaling bifurcates at the level of the receptor (19). The c-Myc-LTβR deletion mutants were examined for their ability to activate NF-κB by electrophoretic mobility shift or NF-κB-dependent reporter assays. HT29.14s cells expressing wild type c-Myc-LTβR displayed constitutive NF-κB binding to the human immunodeficiency virus long terminal repeat κB site, in contrast to empty vector-infected cells, which required treatment with anti-LTβR antibody (Fig. 7*A*, *upper panel*). The constitutive κB binding complex completely shifted with antibodies specific for the 50-kDa subunit (p50, NF-κB) but not the 65-kDa subunit of NF-κB (p65, RelA) (Fig. 7*B*, *upper panel*). The constitutive p50 complex probably represents dimers of p50 (herein referred to as p50 dimers) but may also form a complex with members of the Rel family other than p65. Constitutive activation of p50 by c-Myc-LTβR was lost in the Δ396, indicating that the region controlling activation of p50 dimers is adjacent to the TRAF binding region. As expected, the Δ379 mutant was active, which further supports the idea that this mutant acts as a dominant positive by activating endogenous receptors. The deletion mutant Δ359 did not stimulate formation of constitutive p50 dimers.

Fig. 7. Activation of NF-κB by LTβR. *A*, electrophoretic mobility gel shift assays were performed using a ^{32}P-labeled oligonucleotide probe representing tandem NF-κB binding sites from the human immunodeficiency virus long terminal repeat. Nuclear extracts were prepared from HT29.14s cells containing empty vector (*V*) or cells expressing wild type c-Myc-LTβR (*WT*) or one of the deletion mutants indicated at the *top* of the *lane*.



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Cells were treated 15 min prior to harvest with (+) or without (−) IgG control antibody (*upper panel*), the anti-LTβR monoclonal antibody CBE11 (*upper* and *middle panels*), or TNF (*bottom panel*) as indicated to the *left*. *B*, identification of p50 and p65 subunits. Competition gel shift assays and supershift assays were performed with nuclear extracts (on samples used in *A*) isolated from HT29.14s cells expressing wild type c-Myc-LTβR. As controls, NF-κB-shifted complexes were incubated with a 10-fold molar excess of unlabeled NF-κB (*NFκB*) or mutated NF-κB oligonucleotide (*Mut*) as indicated at the *top*. NF-κB complexes were preincubated with antibody specific for either p65 or p50 subunits of NF-κB prior to electrophoresis. Bands representing the p50 dimers and p50/p65 heterodimers of NF-κB are indicated by *arrows* to the *right*. *C*, LTβR mutants activate NF-κB-dependent luciferase reporter. 293T cells were transfected with 4 μg of the indicated LTβR expression plasmid and 0.25 μg of the NF-κB-luciferase plasmid. Cells were lysed ~36 h after transfection, and the -fold activation in luciferase activity was calculated relative to transfection of 0.25 μg of NF-κB-luciferase plasmid plus 4 μg of empty expression vector.

Treatment of empty vector-infected HT29.14s with anti-LTβR mAb resulted in the formation of NF-κB binding complexes (resolved as a tight doublet) (Fig. 7*A*, *middle panel*), previously shown to supershift with antibodies to p65 and p50 (19). By contrast, anti-LTβR treatment of cells expressing wild type c-Myc-LTβR revealed an additional κB band (compare lanes with vector and wild type in Fig. 7*A*, *middle panel*). Anti-p65 shifted the migration of the upper two complexes, demonstrating the presence of p65 subunit, but had no affect on the fast migrating band (Fig. 7*B*, *lower panel*). A nearly complete mobility shift of these bands occurred with anti-p50 antibody (the small fraction of residual band may represent p65 homodimers). This result indicates that both p50/p65 heterodimers and p50 dimers can be activated in HT29.14s cells by LTβR signaling. Note that treatment of control HT29.14s cells (empty vector alone) with anti-LTβR did not activate the p50 within this short time frame. Furthermore, the presence of p50 in anti-LTβR-activated cells was dependent on the same region (Δ396) of the receptor as unstimulated cells (Fig. 7*A*, *upper panel*). Together, these results indicate that the pattern of NF-κB bands in HT29.14s cells stimulated with anti-LTβR is a composite of the rapidly induced p65/p50 dimers and constitutively formed p50 dimers. Analysis of the κB bands in cells after treatment for 15 min with anti-LTβR showed that formation of p50/p65 heterodimer was inhibited only by the Δ359, further establishing this mutant as a dominant negative that inactivates the function of the endogenous LTβR (Fig. 7*B*, *middle panel*).

As expected, none of these LTβR mutations ablated the formation of p50/p65 complexes induced by TNF (Fig. 7*A*, *lower panel*), and TNF had no detectable effect on the presence of the p50 dimers. However, TNF treatment of HT29.14s cells expressing the wild type c-Myc-LTβR resulted in an enhanced activation of the p50/p65 NF-κB complex (~4-fold). TNF treatment of the remaining c-Myc-LTβR deletion mutants, including the dominant negative mutant c-Myc-LTβRΔ359, also resulted in enhanced activation of the p50/p65 NF-κB heterodimer. These data suggest that TNF-induced activation

of NF-κB may cooperate with the LTβR. Even the Δ359 mutant, which is incapable of activating NF-κB in response to LTβR ligation, can contribute to the enhancement of NF-κB activation by TNF, suggesting that additional sequences in the N-terminal proximal region are responsible for this NF-κB enhancing activity.

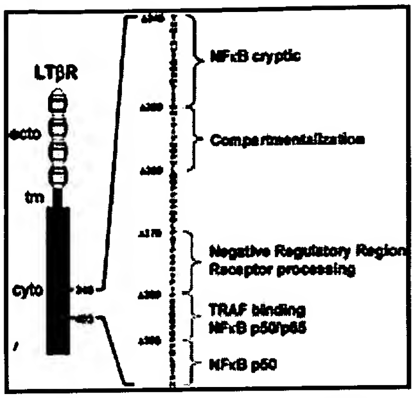
The relatively complex pattern of NF-κB activation in HT29.14s cells prompted us to examine the effect of these mutants in 293T cells. Expression of wild type receptor and mutants Δ418 through Δ396 conferred NF-κB activation as measured by NF-κB-dependent luciferase reporter (Fig. 7C). That Δ396 was functional in this assay but not in HT29.14s is surprising. It is possible that overexpression in 293T can compensate for this mutation. The Δ389 mutant, which deletes the TRAF3 recruitment domain, was inactive. However, significant activation of NF-κB occurred with further truncation of the LTβR including Δ379, Δ369, and Δ359, although with a less robust signal, but was lost with the Δ345 mutant. The lack of a dominant negative effect of Δ359 in this system, as well as the absence of endogenous LT βR for the Δ379 to act through, implicates a TRAF-independent mechanism of NF-κB activation by the LTβR. This result indicates the presence of a cryptic NF-κB activation site that is normally inhibited by the sequence defined by the Δ389 mutant and confirms our suspicion of an additional region that can activate NF-κB. The components involved in this NF-κB pathway are currently being explored.

DISCUSSION

▲ TOP
▲ ABSTRACT
▲ INTRODUCTION
▲ MATERIALS AND METHODS
▲ RESULTS
▪ DISCUSSION
▼ REFERENCES

The LTβR deletion mutants analyzed here define a subregion of the LTβR cytoplasmic domain between Leu[359] and His[403] that functions as a key structural element for receptor compartmentalization and signal transduction (Fig. 8). This sequence encompasses a proline-rich region that is predicted to have an elongated or kinked conformation. Several of the deletion mutants were informative in that they defined discrete sequences with distinct functions including subcellular compartmentalization, TRAF binding, and regulation of cell death and activation of NF-κB. The use of distinct cellular models was invaluable in realizing the effects of these mutations. One significant difference is that HT29.14s cells express endogenous LTβR, whereas 293T cells do not, and this difference clearly affected the behavior of the Δ379 and Δ359 mutants.

Fig. 8. Signaling regions of the LTβR defined by deletion mutants.



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Two distinct binding motifs for TRAF2, -3, and -5 are found in TNFR family members: P*X*QET in CD40 (P*X*Q*X*(T/S) consensus motif in CD27, CD30, 4-1BB, OX40, and Epstein-Barr virus oncoprotein LMP-1) and SKEEC in TNFR2 (a similar motif is also in herpesvirus entry mediator and CD30) (18). Recent crystallographic studies of TRAF2 in complex with TRAF binding peptide from either CD40 (42) or TNFR2 (43) reveal the residues in these peptides that contact TRAF2 are distinct, although the binding affinity and conformation of the peptides are quite similar. The TRAF3 binding region in the LT βR was localized to the sequence ^{389}PEEGDP, which is distinct from both CD40 and TNFR2 motifs. In preliminary results, mutation of both glutamate residues (Glu^{391}-Glu^{392}) in the LTβR was necessary to reduce TRAF3 by 80%, as well as TRAF2 and TRAF5 binding, suggesting that additional residues contribute to the LTβR interaction with TRAF3. Additionally, the activation of NF-κB p50 dimers, presumably a TRAF2- or TRAF5-dependent process, required the sequence ^{396}PPGLSTH adjacent to the TRAF3 binding site, which suggests that the TRAF binding motif in the LTβR may be more complex than the motifs in CD40 or TNFR2.

The Δ379 mutant expressed in HT29.14s cells behaved as a dominant positive mutant that induced ligand-independent cell death and activation of NF-κB in the absence of a functional TRAF binding site. As shown by cotransfection with wild type receptor, Δ379 formed complexes with wild type LTβR, which recruited TRAF3, and caused colocalization to the same subcellular compartment. Thus, the Δ379-HT29.14s cells required only the requisite signal from IFN-γ to induce cell death, indicating that the Δ379-wild type complex is actively signaling. Here, we would envision that the Δ379 sequence may provide a binding site for a regulatory protein that normally blocks spontaneous aggregation, perhaps analogous to SODD for TNFR1 (44, 45). In this regard, expression of wild type LTβR in HT29.14s cells, although only modestly increasing surface expression, enhanced the responsiveness to cell death, recruitment of TRAF3, and NF-κB activation. These findings are consistent with the idea that exceeding a certain threshold of receptor density increases the probability that receptors will spontaneously aggregate and initiate limited signal transduction in the absence of ligand. Indeed, modest overexpression was sufficient for activation of p50 complexes of NF-κB but not the p50/p65 complex. Furthermore, HT29.14s cells still required ligand to induce cell death, indicating that additional

mechanism(s) prevented full receptor activation, which implicates the region spanning [379]PATPEPPYPI as a critical regulatory sequence.

The enhanced responsiveness of HT29.14s cells expressing wild type LTβR or the other mutants (except for Δ359) and the insensitivity of 293T cells to death induced by LTβR precluded an unambiguous test to define the role of the TRAF binding site in the cell death pathway. However, previous results with dominant negative forms of TRAF3 indicate that recruitment and oligomerization of TRAF3 is important to specifically activate the LTβR death pathway (29, 46). How TRAF3 propagates the signal to the death pathway remains to be elucidated.

The Δ359 mutant functioned as a dominant negative mutant in HT29.14s cells that suppressed the signaling action of endogenous LTβR for cell death and NF-κB activation. This mutant was expressed at increased levels on the cell surface, potentially mediating its dominant negative effect as a decoy receptor (*i.e.* retaining ligand binding but lacking signaling capacity). An alternative or contributing mechanism is suggested from confocal microscopy, which showed that the Δ359 mutant effectively blocked accumulation of wild type LTβR into the perinuclear compartments. This result implies that entry into this compartment may be necessary for signal transduction. That both dominant positive and negative mutants can interact with wild type receptors indicates that LTβR contains a self-association domain that is membrane-proximal to Δ359. Recent studies by Wu *et al.* (46) using large deletions mapped a self-association domain to amino acids 324-377, consistent with our results.

LTβR initiates transcription by activating NF-κB that is dependent on TRAF5 or TRAF2. However, recent evidence from gene knockouts challenges this idea. TRAF2-, TRAF3-, or TRAF5-deficient mice exhibit secondary lymphoid tissue development, a phenotype that is observed by deletion of LTβR (8). Although TRAF6 does not appear to bind LTβR, mice deficient in TRAF6 fail to develop lymph nodes, a phenotype that is thought to be linked to the osteoclast differentiation factor (OPGL/RANK) pathway (47). The alymphoplasia (*aly*) mouse, which lacks lymph nodes, results from mutant NF-κB-inducing kinase (48). TRAF2 and -5 can act as adapters coupling receptors to NF-κB-inducing kinase, which then activates IκB kinases, leading to activation of NF-κB. TRAF2 and TRAF5 could be functionally redundant as adapters involved in signaling lymph node development, and here, a double knockout should reveal the expected phenotype. The finding that Δ379, Δ369, and Δ359 initiated NF-κB-dependent transcription in 293T cells suggests that another TRAF-independent NF-κB-activating pathway is operative in the LTβR. This effect was revealed only after truncation of the TRAF binding site (defined by Δ389), implicating TRAF3 as a possible inhibitor of this cryptic NF-κB-activating site. The possibility exists that this cryptic TRAF-independent, NF-κB activation mechanism is involved in activating NF-κB-inducing kinase and subsequent signaling for lymphoid organogenesis.

Different subregions of the LTβR appear to be responsible for activating distinct forms of NF-κB. The [396]PPGLSTPH sequence is crucial for the activation of constitutive p50 complexes as defined by the Δ396 mutant. By contrast, the NF-κB luciferase assay indicated that Δ389, which lacks the TRAF3 binding sequence, was essential for activating NF-κB. Because these sites are adjacent, the mutations may have disrupted the full site, creating high and low affinity sites not revealed by current TRAF binding assays, and thus the loss of constitutive p50 activation may reflect the need for a higher affinity

interaction. The significance of LTβR activation of p50 dimers is unclear, because p50 partners with several other Rel family members that can act as either suppressors or activators of transcription in tissue-specific contexts (49). However, distinct p50 complexes formed have different transactivating potentials that may in turn contribute to activation of specific subsets of genes.

The investigation of these mutants has revealed several new features of the LTβR signal transduction pathways that illuminate the molecular steps involved in physiologic roles of this receptor.

ACKNOWLEDGEMENTS

We thank Jeff Browning for cytokines, Michael Lai and Laura Williams-Abbott for the glutathione *S*-transferase fusion analysis, George Mosialos and Elliott Kieff for the TRAF3-FLAG cDNA, and Cheryl McLaughlin for the excellent assistance with figures and manuscript preparation.

FOOTNOTES

* This work was supported in part by U. S. Public Health Service, National Institutes of Health (NIH) Grants CA69381 and AI03368, and American Cancer Society Grant IM663 (to C. F. W.). This work was also supported by University-wide AIDS Research Program Fellowship F95-R-008 (to W. F.) and NIH Training Grants T32AI07469 (to A. G.) and T32AG00252 (to C. D. B.). This is publication #333 from the La Jolla Institute for Allergy and Immunology.The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Gemini Science, Inc., San Diego, CA 92121.

§ To whom correspondence should be addressed: Division of Molecular Immunology, La Jolla Institute for Allergy and Immunology, 10355 Science Center Dr., San Diego, CA 92121. Tel.: 619-558-3500; Fax: 619-558-3525; E-mail: carl_ware@liai.org.

ABBREVIATIONS

The abbreviations used are: LT, lymphotoxin; LTβR, LTβ receptor; IFN-γ, interferon-γ; mAb, monoclonal antibody; PCR, polymerase chain reaction; TNF, tumor necrosis factor; TNFR, TNF receptor; TRAF, TNFR-associated factor; PBS, phosphate-buffered saline; BSA, bovine serum albumin; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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▲ TOP
▲ ABSTRACT

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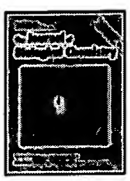

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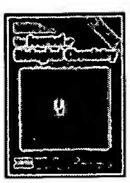

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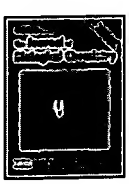

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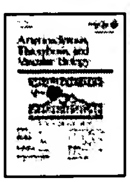

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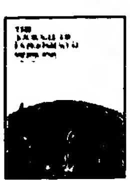

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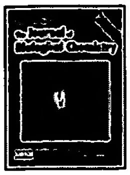

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